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(71) Applicant *(for all designated States except AT US)*: NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH–4058 Basel (CH).

(71) Applicant *(for AT only)*: NOVARTIS–ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B.H. [AT/AT]; Brunner Strasse 59, A–1230 Vienna (AT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HEIFETZ, Peter, Bernard [US/US]; 3916 Sturbridge Drive, Durham, NC 27713 (US). GOFF, Stephen, Arthur [US/US]; 1040 Calle Anacapa, Encinitas, CA 92024 (US). TUTTLE, Annmarie, Bloom [US/US]; 618 Benfield Court, Garner, NC 27529 (US). GRIOT–WENK, Monika, Else [CH/CH]; Schönenbühl, CH–3179 Kriechenwil (CH).

(74) Agent: BECKER, Konrad; Novartis AG, Corporate Intellectual Property, Patent & Trademark Dept., CH–4002 Basel (CH).

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(54) Title: THERAPEUTICALLY ACTIVE PROTEINS IN PLANTS

(57) Abstract

The present invention discloses transgenic plants expressing therapeutically active proteins, preferably from their plastid genome or targeted to the vacuole. The present invention also describes the administration of such transgenic plants to a host in need thereof for the prevention or treatment of diseases. In a preferred embodiment, such plants or matter derived from such plants is administered orally to a host.

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THERAPEUTICALLY ACTIVE PROTEINS IN PLANTS

The present invention generally relates to transgenic plants expressing therapeutically active proteins. In particular, the present invention relates to plants expressing therapeutically active proteins in subcellular organelles, preferably in vacuoles or more preferably in plant plastids. The present invention also relates to the therapeutic uses of such transgenic plants.

A wide variety of diseases afflict animals, including humans, pets and livestock. These diseases not only cause great suffering, but they also translate into major economic losses. Sick workers are unable to work or have a reduced performance, and agricultural yields can be dramatically decreased by diseases affecting farm animals. Every human civilization has reacted to diseases with its own medications and although many diseases have been and are still combated successfully by current medicines, many other diseases are still resisting treatments or can be only partially treated.
Among the problems impacting the treatment of diseases are the costs of a large number of medications or their limited supply. Thus, in some cases, only a small number of patients can be efficiently treated, particularly in developing countries, and expensive treatments are a heavy burden on the healthcare systems throughout the world. Moreover, in the agricultural field, some diseases resulting in lower yields are inadequately treated because the costs greatly exceed the benefits gained by an efficient treatment. An important contribution to the high cost of some medications is the lack of economic production methods, particularly for protein or peptide based medications. Another sometimes overlooked but crucial problem of some current medicines resides in the administration of therapeutically effective amounts of the medicine to the desired host's organs or body parts. Indeed, in some cases, currently available delivery methods are not completely satisfactory, as for example, for the treatment of allergies or autoimmune diseases. An additional problem connected with medications is their transport, particularly in hot climates, which requires expensive refrigeration and expensive formulations, thus further increasing costs. The availability of a wide array of medicines without having to incur expensive transportation or administration costs would be of great advantage.
Plants are attractive candidates for the expression of therapeutically active proteins because they may be edible and have high biomass yields. Expression of therapeutically

active proteins in cellular compartments sequestered away from cytoplasmic proteases is a particularly appealing way for producing therapeutically active compounds in plants. Overall, there is therefore a constant and unfulfilled need for novel medications, available in large and inexpensive supply, which can be administered to a patient or host through a wide variety of methods and which are easily accessible throughout the world.

The present invention addresses the need for large and inexpensive supplies of medications, in particular protein-based medications, for the prevention or treatment of diseases. Accordingly, the present invention provides transgenic plants capable of expressing therapeutically active proteins, in particular transgenic plants capable of expressing therapeutically active proteins in subcellular organelles, preferably in vacuoles or more preferably in plant plastids. The plants of the present invention are particularly useful in the context of the suppression or reduction of an undesired immune response. Proteins expressed in transgenic plants can be administered to a host by a wide range of delivery methods, preferably orally. For example, in some cases and as described *infra*, plants or plant material containing therapeutically active proteins can be ingested orally without prior extraction or purification, or with minor extraction or purification. Such prevention or treatment of diseases through the diet is convenient and reduces the cost of a medication considerably. Alternatively, the proteins expressed in transgenic plants can be extracted and administered using methods well known in the medical arts.
In particular, the present invention relates to transgenic plants expressing therapeutically active proteins from their plastid genome. Expression levels in plastids according to the present invention regularly exceed those of nuclear expressed transgenes. Such high levels of expression result in high concentrations of protein per gram of plant tissue thereby fulfilling a long-felt but heretofore unfulfilled need by allowing the use of plants or plant material derived from such plants in a wide range of therapeutic applications. Furthermore, transgene expression levels are stable over time due to the absence of gene silencing, and position effect variation is not an issue because of the insertion of the transgene at a precise, targeted position on the plastid genome by homologous recombination Also, proteins expressed within plant plastids remain sequestered in the organelle and are thus conveniently protected from degradation, in particular from degradation in the digestive system when the plant is ingested orally. Sequestration in the organelle also prevents plastid-expressed transgenes from interacting with the cytoplasmic environment. This

feature is also essential if the protein expressed in plants also shows activity against some components of the plant. Also, inducible plastid expression systems are available (see WO 98/11235), allowing the restriction of the expression of the transgene to a desired time-point and thus limiting potentially detrimental effects of high transgene expression levels over long periods of time. Similarly, proteins expressed from the nuclear genome which are targeted to other subcellular compartments such as the vacuole are also protected from degradation or from adverse interactions with the cytoplasmic environment.
The present invention also provides therapeutic compositions comprising plant matter derived from such transgenic plants, novel methods for expressing therapeutically active proteins and novel methods for improving the condition of a wide range of hosts including human, livestock, pets and other animals. Methods for administering plant-derived therapeutic agents are also provided.

The invention therefore provides:
A plant comprising in its plastid genome at least one DNA molecule encoding at least one protein that is therapeutically active when administered to a host, preferably a host in need thereof, in a therapeutically effective amount, wherein said plant is capable of expressing said protein or proteins. In a preferred embodiment, the therapeutically active protein accumulates in the plastids of said plant. In another preferred embodiment, the protein is expressed in edible parts of the plants. In another preferred embodiment, the protein is administered orally to the host. In another preferred embodiment, the host is a vertebrate, preferably a mammal, more preferably human, bovine, ovine, porcine, canine or feline. In a further preferred embodiment, the therapeutically active protein is an antigen, preferably an immunologically active antigen. Accordingly, the present invention provides a plant comprising in its plastid genome a DNA molecule encoding an antigen, preferably an antigen that is immunologically active, wherein the plant is capable of expressing the antigen. Preferably, the antigen is expressed in the plant and, more preferably, an immune response to the antigen is reduced after ingestion by the host of the plant.
In another preferred embodiment, the antigen is capable of suppressing or reducing the immune response of the animal to the antigen, preferably by inducing tolerance of the host to the antigen, such as e.g. by contact or uptake by the gut mucosa. A preferred antigen is an allergen, preferably an airborne allergen, preferably a pollen allergen. Examples of preferred allergens are *Der f I, Der f II, Der p I and Der p II, Can f II, Lol p V, Sor h I, Amb a*

I, preferably *Amb a I.1, Amb a II, Aln g I, Cor a I, Bet v I, Fel d I,* or *rAed a 1* and *rAed a 2*. For example, an allergen expressed in a plant of the present invention is not glycosylated. Another preferred antigen is an autoantigen, such as collagen, preferably type I or type III collagen, type II collagen, myelin basic protein, myelin proteolipid protein, interphotoreceptor binding protein, acetylcholine receptor, an S-antigen, insulin, glutamic acid dehydrogenase, an islet cell-specific antigen or thyroglobulin, or a transplantation antigen, such as an allo- or xeno-transplantation antigen, for example a MHC protein, preferably a MHC class II protein, preferably an a or a b chain. In an alternate preferred embodiment, an immunologically active antigen is capable of inducing immunization of the animal against the antigen. In yet another preferred embodiment, the therapeutically active protein is a blood protein, a hormone, a growth factor, a cytokine, an enzyme, a receptor, a binding protein, an immune system protein, a translation or transcription factor, an oncoprotein or proto-oncoprotein, a milk protein, a muscle protein, a myeloprotein, a neuroactive peptide or a tumor growth suppressing protein or peptide, for example angiostatin or endostatin, both of with inhibit angiogenesis. In another preferred embodiment of the invention, the therapeutically active protein is an anti-sepsis peptide, such as BPI (bactericidal permeability-increasing protein). In yet another embodiment, the immune system protein is an antibody. The DNA molecule according to the invention is operably linked to a promoter capable of expressing said DNA molecule in the plastids of said plant. In a preferred embodiment the promoter is a *clpP* promoter, in another preferred embodiment a 16S r-RNA gene promoter. In a particularly preferred embodiment of the invention, said promoter is a transactivator-mediated promoter, particularly a T7 gene 10 promoter. In addition, the invention provides a plant further comprising a heterologous nuclear expression cassette comprising a DNA sequence encoding a transactivator. In a preferred embodiment the transactivator is a T7 polymerase. The invention further provides a plant comprising in its nuclear genome a DNA molecule encoding a protein that is therapeutically active when administered to a host in need thereof in a therapeutically effective amount, wherein said therapeutically active protein is selected from the group consisting of the mosquito allergens *rAed a 1* and *r Aed a 2*, bactericidal permeability-increasing protein (BPI) and the pollen allergens *Amb a I, Amb a I.1, Amb a II*, Amb t V, *Aln g I, Cor a I, Lol p V, Sor h* and *Bet v I*.

The invention also provides a plant comprising in its nuclear genome a DNA molecule encoding a protein that is therapeutically active when administered to a host in need thereof in a therapeutically effective amount, wherein said therapeutically active protein is targeted

to a subcellular organelle of said plant. In a preferred embodiment, the therapeutically active protein is targeted to the vacuole. Said therapeutically active protein which is targeted to the vacuole preferably is selected from a group consisting of allergens such as *Der f I, Der f II, Der p I and Der p II, Can f II, Lol p V, Sor h I, Amb a I,* preferably *Amb a I.1, Amb a II,* Amb t V, *Aln g I, Cor a I, Bet v I, Fel d I,* or *rAed a 1* and *rAed a 2*, autoantigens such as collagen, preferably type I or type III collagen, type II collagen, myelin basic protein, myelin proteolipid protein, interphotoreceptor binding protein, acetylcholine receptor, an S-antigen, insulin, glutamic acid dehydrogenase, an islet cell-specific antigen or thyroglobulin, or a transplantation antigen, such as an allo- or xeno-transplantation antigen, for example a MHC protein, preferably a MHC class II protein, preferably an a or a b chain, or from the group consisting of a blood protein, a hormone, a growth factor, a cytokine, an enzyme, a receptor, a binding protein, an immune system protein, a translation or transcription factor, an oncoprotein or proto-oncoprotein, a milk protein, a muscle protein, a myeloprotein, a neuroactive peptide or a tumor growth suppressing protein or peptide, for example angiostatin or endostatin, both of with inhibit angiogenesis. In another preferred embodiment the therapeutically active protein targeted to the vacuole is an anti-sepsis peptide, such as BPI (bactericidal permeability-increasing protein).
In a further preferred embodiment, the plant according to the invention is an edible plant. In another preferred embodiment the plant is a dicotyledonous plant, preferably tobacco, tomato, soybean or spinach. In an alternate embodiment, the plant is a monocotyledonous plant, preferably maize or rice.
In a further preferred embodiment, the expression of the protein in the plant is regulatable, preferably chemically regulatable. Alternatively, the expression of the protein is constitutive, tissue specific or developmentally regulated.
This also includes the seed for such a plant, which seed is optionally treated (e.g., primed or coated) and/or packaged, e.g. placed in a bag or other container with instructions for use.
The host according to the invention is a vertebrate, particularly a mammal, more particularly a human, bovine, ovine, porcine, canine or feline.

The invention further provides:
A composition comprising a plant according to the invention or plant matter derived from said plant, wherein said composition comprises a therapeutically effective amount of said

protein and a composition wherein said plant is processed prior to being administered to said host.

The invention further provides methods wherein:
- a host in need thereof is being administered a composition according to the invention in an amount effective to improve the condition of said host
- said composition is administered orally to said host
- said protein is an antigen, whereby an immune response of said host against said antigen is suppressed or reduced
- said antigen particularly is an allergen, an autoantigen or a transplantation antigen

The invention also provides a method of treating or preventing a disease, comprising administering to an host in need thereof a therapeutically effective amount of a plant according to the invention or plant matter derived from said plant.
In a specific embodiment said disease is an allergy, an autoimmune disease or the rejection of a transplantation. In yet another specific embodiment said therapeutically effective amount is administered orally to said host.

The invention further provides:
A plant of the present invention for use as a pharmaceutical, preferably to treat or prevent diseases, e.g. allergies, autoimmune diseases or transplantations, for example by induction of tolerance, e.g. oral tolerance, in a patient in need thereof, or for the immunization of a host.

The invention further provides:
A plant of the present invention for use as a medical food, preferably to treat or prevent diseases, e.g. allergies, autoimmune diseases or transplantations, for example by induction of tolerance, e.g. oral tolerance, in a patient in need thereof, or for the immunization of a host.

The invention further provides:
A plastid transformation vector comprising a DNA molecule encoding a protein that is therapeutically active when administered to a host in need thereof in a therapeutically

effective amount, wherein said DNA molecule is operably linked to a promoter capable of directing the expression of said DNA molecule in a plant plastid. In a preferred embodiment, the protein accumulates in the plastids of said plant. In another preferred embodiment, a promoter in the plastid transformation vector is a *clpP* promoter, a 16S r-RNA gene promoter, a *psbA* promoter, a *rbcL* promoter or a transactivator-mediated promoter regulated by a nuclear transactivator (e.g., the T7 gene 10 promoter when the transactivator is T7).

A plastid comprising a transformation vector as described above.

A plant cell comprising a plastid as described above, wherein said plant cell is capable of producing said protein.

The invention furthermore provides:

A plant comprising

a heterologous nuclear expression cassette comprising a promoter, e.g., an inducible promoter, e.g., a wound-inducible or chemically-inducible promoter, for example the tobacco PR-1a promoter, or a tissue- or organ-specific promoter or a developmentally regulated promoter, operably linked to a DNA sequence coding for a transactivator (preferably a transactivator not naturally occurring in plants, preferably a RNA polymerase or DNA binding protein, e.g., T7 RNA polymerase), said transactivator being optionally fused to a plastid targeting sequence, e.g., a chloroplast targeting sequence (e.g., a plant expressible expression cassette); and

a heterologous plastid expression cassette comprising a transactivator-mediated promoter regulated by the transactivator (e.g., the T7 gene 10 promoter when the transactivator is T7 RNA polymerase) and operably linked to a DNA molecule encoding a therapeutically active protein of the present invention;

also including the seed for such a plant, which seed is optionally treated (e.g., primed or coated) and/or packaged, e.g. placed in a bag or other container with instructions for use.

The invention further provides:

A composition comprising a plant or plant matter as described above, wherein said composition comprises an amount of said protein that is therapeutically effective when administered to a host in need thereof. In a preferred embodiment, the plant matter is processed prior to being administered to the host. The processing is preferably a type of

processing routinely used in the food or feed industry. In another preferred embodiment, a composition of the present invention comprises an amount of an antigen that is immunologically effective.

The invention further provides:
Pharmaceutical compositions comprising a plant or plant matter of the present invention and medical food compositions comprising a plant or plant matter of the present invention.

The invention also further provides:
A method comprising transforming the plastid genome of a plant with a transformation vector as described above, preferably further comprising expressing a therapeutically active protein in said plant. In a preferred embodiment, the therapeutically active protein is an antigen, preferably an immunologically active antigen.

The invention also further provides:
A method comprising administering to a host in need thereof a composition as described above in an amount effective to improve the condition of the host. Preferably, the method comprises oral administration of the composition to the host.

The invention further provides:
A method of treating or preventing a disease, e.g. allergies, autoimmune diseases or rejections of transplantations, for example by induction of tolerance in a host in need thereof, or for the immunization of a host, comprising administering a therapeutically effective amount of a plant of the present invention or plant matter derived thereof to the host.

The present invention further provides:
The use of a plant of the present invention in the manufacture of a medication for the treatment or prevention of diseases, e.g. for the treatment of allergies, autoimmune diseases or transplantations, for example by induction of tolerance in a host in need thereof, or for the immunization of a host.

The present invention further provides:

The use of a plant of the present invention in the manufacture of a medical food for the treatment or prevention of diseases, e.g. for the treatment of allergies, autoimmune diseases or transplantations, for example by induction of tolerance in a host in need thereof, or for the immunization of a host.

The present invention further provides:
The use of a plant of the present invention for the production of an antigen for determination of immunological activity.

The present invention further provides:
An antibody specific for an antigen expressed in a plant of the present invention.
An antibody that interferes with the binding of an antibody specific for an antigen expressed in a plant of the present invention with the expressed antigen.

The present invention further provides:
A food product comprising an edible portion of a plant comprising a DNA molecule according to the invention, wherein said food product is therapeutically active when administered to a host in need thereof in a therapeutically effective amount.

The present invention further provides:
An agricultural product derived from a plant or plant part comprising a DNA molecule according to the invention, wherein said agricultural product is therapeutically active when administered to a host in need thereof in a therapeutically effective amount.

The present invention also further provides:
All novel products, processes, and utilities as described herein.

DEFINITIONS

In a broad sense, a “therapeutically active protein” contributes to the condition of a host in a positive manner when administered to the host in a therapeutically effective amount. A therapeutically active protein has healing, curative or palliative properties against a disease and may be administered to ameliorate, relieve, alleviate, reverse, or lessen the severity of

the disease. A “therapeutically active protein” also has prophylactic properties and is used to prevent the onset of a disease or to lessen the severity of such disease or pathological condition when it does emerge. The term “therapeutically active protein” comprises an entire protein or peptide, or therapeutically active fragments thereof. It also comprises therapeutically active analogs of the protein or peptide, or analogs of fragments of the protein or peptide. The term “therapeutically active protein” also refers to a plurality of proteins or peptides that act cooperatively or synergistically to provide a therapeutic benefit.

An "analog" of a therapeutically active protein includes proteins that are so structurally related to the protein that they possess the same biological activity as the protein.

An “immune response” is referred to as the physiological responses stemming from the activation of the immune system by antigens. In the present invention, the immune response may be suppressed through the induction of tolerance based on exposure to the antigen, particularly when the antigen is orally administered.

“Immunologically active” means herein capable of modulating the immune system, for example by stimulating an immune response or by suppressing or reducing an immune response or an inflammatory condition.

An “antigen” is a substance which interacts with the immune system, preferably with products of specific humoral or cellular immunity to stimulate an “immune response”. An antigen is preferably a polypeptide and is a “therapeutically active protein”. An antigen comprises the polypeptide in its entirety or a portion of the polypeptide. Such portion of the polypeptide is for example an epitope or an antigenic determinant of the antigen. An antigen may comprise one or more than one epitopes or antigenic determinants. An antigen also comprises "analogs" of the antigen including molecules that are so structurally related to the antigen that they possess the same biological activity as the antigen, i.e. the same immunological activity. In the context of the present invention, an antigen encompasses e.g. allergens, autoantigens and transplantation antigens.

An “epitope” is a portion of an antigen that determines its capacity to combine with the specific combining site of a corresponding antibody in a antigen-antibody interaction.

An “antigenic determinant” is the portion of an antigen that determine the specificity of the immune response stimulated by the antigen.

An “adjuvant” is a substance, preferably oily, which, when mixed and administered with an antigen, in particular when mixed and injected with an antigen, nonspecifically enhances an immune response to the antigen. A typical adjuvant is the complete Freund’s adjuvant (CFA) or the incomplete Freund’s adjuvant (Lando et al. (1981) J.P. immunol. 126: 1526).

An "autoantigen" is any substance normally found within an animal that, in an abnormal situation such as an autoimmune disease, is no longer recognized as part of the animal itself by the immune system of that animal, and is therefore attacked by the immune system as though it were a foreign substance.

An “allergen” is an antigen that induces an allergic reaction of a host.

“Immunization” of a host against a pathogen or a toxin is one aspect of stimulating an immune response and refers herein to as a protection of the host against the pathogen or toxin by an induction of the immune system. Preferably both an immediate immune response and an immunological memory are induced, preferably providing for immediate and a long-term protection of the host. An antigen of the host of the toxin are used for the immunization. A vaccination is also encompassed by the term immunization.

"Administration" of therapeutically active protein to a host in need thereof is intended as providing the therapeutically active protein to such host in a manner which retains the therapeutic effectiveness of such protein for a length of time sufficient to provide a desired beneficial effect to such host.

“Oral administration” of a therapeutically active protein means primarily administration by way of the mouth, preferably by eating, but also intends to include any administration which provides such proteins to the host's stomach or digestive tract. In a preferred embodiment, oral administration results in contact of the therapeutically active protein with the gut mucosa.

A “host” is an animal to whom a therapeutically active protein of the present invention is administered. The term "animal" covers all life forms that have an immune system, including humans, bovines, ovines, porcines, canines or felines.

“Food” or “food product” means herein liquid or solid food or foodstuff or feedstuff and is a plant, plant part or plant matter derived thereof which is ingested by humans and other animals. This term is intended to include raw plants or plant material which may be fed directly to humans and other animals or any processed plant matter together with a nutritional carrier which is fed to humans and other animals. Materials obtained from a plant are intended to include any component of a plant which is eventually ingested by a human or other animal.

A “medical food” comprises a composition that is eaten or drunk by a host and has a therapeutic effect on the host. A medical food comprises for example a plant of the present invention or plant matter derived thereof. Medical food may be ingested alone or may be administered in combination with a pharmaceutical composition well-known in the medical arts. A medical food also comprises the equivalent feed-stuff for non-human animals.

“Agricultural product“ as used herein refers to a plant that is eaten as a whole such as alfalfa sprouts, radish sprouts, wheat sprouts and the like or to an edible portion of a plant which is consumed by humans in either raw or cooked form. The edible portion may be a root, such as rutabaga, beet, carrot, and sweet potato; a tuber or storage stem, such as potato, Jerusalem artichoke and taro; the stem, as in asparagus and kohlrabi; a bud, such as brussels sprouts; a bulb, such as onion and garlic; a petiole or leafstalk, such as celery and rhubarb; a leaf, such as cabbage, lettuce, parsley and spinach; an immature flower, such as cauliflower, broccoli and artichoke; a seed; the immature fruit, such as eggplant, cucumber, and sweet corn (maize); or the mature fruit, such as tomato, pepper, apple, pear, banana, orange, berries and the like.

A "plant" refers to any plant particularly to seed plants.

“Plant cell” refers to the structural and physiological unit of the plant, comprising a protoplast and a cell wall. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, a plant tissue, or a plant organ.

"Plant material" refers to leaves, stems, roots, seeds, flowers or flower parts, fruits, pollen, pollen tubes, ovules, embryo sacs, egg cells, zygotes, embryos, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant.
"Plant matter" refers to any part of a plant at any stage of development, preferably such parts that can be administered orally. Plant matter includes edible parts of a plant, such as leaves, seeds, fruits, tubers, or other plant parts that can be ingested raw or unprocessed. Plant matter also includes isolated fractions of the plants, such as subcellular organelles, e.g. plastids or vacuoles. Plant matter also includes parts of a plant that have been subjected to various types of processing steps, in particular processing steps commonly used in the food or feed industry. Such steps include but are not limited to concentration or condensation of the solid matter of the plant to form for example a pellet, production of a paste, drying, or lyophilization, or by fragmentation of the plant to various extents by cutting or grinding, or by extraction of the liquid part of the plant to produce a soup, a syrup or a juice. A processing step can also include cooking the plant or plant matter.

"Expression" refers to the transcription and/or translation of an endogenous gene or a transgene in plants. In the case of antisense constructs, for example, expression may refer to the transcription of the antisense DNA only.

"Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is optionally operably linked to 3' sequences, such as 3' regulatory sequences or termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA that, in the sense or antisense direction, inhibits expression of a particular gene, e.g., antisense RNA. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that the nucleotide sequence is comprised of more than one DNA sequences of distinct origin which are fused together by recombinant DNA techniques resulting in a nucleotide sequence which does not occur naturally, and which particularly does not occur in the plant to be transformed. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression.

Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular DNA sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue or organ or stage of development. A nuclear expression cassette is usually inserted into the nuclear genome of a plant and is capable of directing the expression of a particular nucleotide sequence from the nuclear genome of said plant. A plastid expression cassette is usually inserted in to the plastid genome of a plant and is capable of directing the expression of a particular nucleotide sequence from the plastid genome of said plant. In the case of a plastid expression cassette, for expression of the nucleotide sequence from a plastid genome, additional elements, i.e. ribosome binding sites, or 3' stem-loop structures that impede plastid RNA polyadenylation and subsequent degradation may be required.

"Gene" refers to a coding sequence and associated regulatory sequences wherein the coding sequence is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Examples of regulatory sequences are promoter sequences, 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns.

"Heterologous" as used herein means of different natural or of synthetic origin. For example, if a host cell is transformed with a nucleic acid sequence that does not occur in the untransformed host cell, that nucleic acid sequence is said to be heterologous with respect to the host cell. The transforming nucleic acid may comprise a heterologous promoter, heterologous coding sequence, or heterologous termination sequence. Alternatively, the transforming nucleic acid may be completely heterologous or may comprise any possible combination of heterologous and endogenous nucleic acid sequences. Similarly, heterologous refers to a nucleotide sequence derived from and inserted into the same natural, original cell type, but which is present in a non-natural state, e.g. a different copy number, or under the control of different regulatory elements.

“Marker Gene” a gene encoding a selectable or screenable trait.

A regulatory DNA sequence is said to be “operably linked to” or “associated with” a DNA sequence that codes for an RNA or a protein if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence.

“Regulatory elements” refer to sequences involved in conferring the expression of a nucleotide sequence. Regulatory elements comprise a promoter operably linked to the nucleotide sequence of interest and, optionally, 3’ sequences, such as 3’ regulatory sequences or termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

“Subcellular organelles” refers to intracellular organs of characteristic structure and function. Subcellular organelles are for example vacuoles, plastids, mitochondria, the cell nucleus, the endoplasmic reticulum or the plasma membrane.

"Homoplasmic" refers to a plant, plant tissue or plant cell wherein all of the plastids are genetically identical. This is the normal state in a plant when the plastids have not been transformed, mutated, or otherwise genetically altered. In different tissues or stages of development, the plastids may take different forms, e.g., chloroplasts, proplastids, etioplasts, amyloplasts, chromoplasts, and so forth.

A “promoter” refers to a DNA sequence that initiates transcription of an associated DNA sequence. The promoter region may also include elements that act as regulators of gene expression such as activators, enhancers, and/or repressors.

An "inducible promoter" is a promoter which initiates transcription only when the plant is exposed to some particular external stimulus, as distinguished from constitutive promoters or promoters specific to a specific tissue or organ or stage of development. Particularly preferred for the present invention are chemically-inducible promoters and wound-inducible promoters. Chemically inducible promoters include plant-derived promoters, such as the promoters in the systemic acquired resistance pathway, for example the PR promoters, e.g., the PR-1, PR-2, PR-3, PR-4, and PR-5 promoters, especially the tobacco PR-1a promoter

and the *Arabidopsis* PR-1 promoter, which initiate transcription when the plant is exposed to BTH and related chemicals. *See* US Patent 5,614,395, incorporated herein by reference, and WO 98/03536, incorporated herein by reference. Chemically-inducible promoters also include receptor-mediated systems, e.g., those derived from other organisms, such as steroid-dependent gene expression, for example the glucocorticoid, progesterone and estrogen receptor systems, copper-dependent gene expression, such as that based on ACE1, tetracycline-dependent gene expression, the Lac repressor system and the expression system utilizing the USP receptor from *Drosophila* mediated by juvenile growth hormone and its agonists, described in WO 97/13864, incorporated herein by reference, as well as systems utilizing combinations of receptors, e.g., as described in WO 96/27673, incorporated herein by reference. Additional chemically-inducible promoters include elicitor-induced promoters, safener-induced promoters as well as the alcA/alcR gene activation system that is inducible by certain alcohols and ketones (WO 93/21334; Caddick et al. (1998) Nat Biotechnol 16:177-180, the contents of which are incorporated herein by reference). Wound inducible promoters include promoters for proteinase inhibitors, e.g., the proteinase inhibitor II promoter from potato, and other plant-derived promoters involved in the wound response pathway, such as promoters for polyphenyl oxidases, LAP and TD. *See generally*, C. Gatz, "Chemical Control of Gene Expression", Annu. Rev. Plant Physiol. Plant Mol. Biol. (1997) 48: 89-108, the contents of which are incorporated herein by reference.

A "transactivator" is a protein which, by itself or in combination with one or more additional proteins, is capable of causing transcription of a coding region under control of a corresponding transactivator-mediated promoter. Examples of transactivator systems include bacteriophage T7 gene 10 promoter, the transcriptional activation of which is dependent upon a specific RNA polymerase such as the phage T7 RNA polymerase. The transactivator is typically an RNA polymerase or DNA binding protein capable of interacting with a particular promoter to initiate transcription, either by activating the promoter directly or by inactivating a repressor gene, e.g., by suppressing expression or accumulation of a repressor protein. The DNA binding protein may be a chimeric protein comprising a binding region (e.g., the GAL4 binding region) linked to an appropriate transcriptional activator domain. Some transactivator systems may have multiple transactivators, for example promoters which require not only a polymerase but also a specific subunit (sigma factor) for

promoter recognition, DNA binding, or transcriptional activation. The transactivator is preferably heterologous with respect to the plant or to the subcellular organelle or component of the plant cell in which induction is effected.

A "minimal promoter" comprises promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation. In the presence of suitable upstream activating sequences fused to the minimal promoter and of corresponding transcription factor, the minimal promoter functions to permit transcription.

"Recombinant DNA technology" refers to procedures used to join together DNA sequences as described, for example, in Sambrook et al., 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

A "screenable marker gene" refers to a gene whose expression does not confer a selective advantage to a transformed cell, but whose expression makes the transformed cell phenotypically distinct from untransformed cells.

A "selectable marker gene" refers to a gene whose expression in a plant cell gives the cell a selective advantage. The selective advantage possessed by the cells transformed with the selectable marker gene may be due to their ability to grow in the presence of a negative selective agent, such as an antibiotic or a herbicide, compared to the growth of non-transformed cells. The selective advantage possessed by the transformed cells, compared to non-transformed cells, may also be due to their enhanced or novel capacity to utilize an added compound as a nutrient, growth factor or energy source. Selectable marker gene also refers to a gene or a combination of genes whose expression in a plant cell gives the cell both, a negative and a positive selective advantage.

"Synthetic" refers to a nucleotide sequence comprising structural characters that are not present in the natural sequence. For example, an artificial sequence that resembles more closely the G+C content and the normal codon distribution of dicot and/or monocot genes is said to be synthetic.

"Transformation" refers to introduction of a nucleic acid into a cell. In particular, the stable integration of a DNA molecule into the genome of an organism of interest.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID No:1	oligonucleotide
SEQ ID No:2	oligonucleotide
SEQ ID No:3	oligonucleotide
SEQ ID No:4	oligonucleotide
SEQ ID No:5	oligonucleotide
SEQ ID No:6	oligonucleotide
SEQ ID No:7	oligonucleotide
SEQ ID No:8	oligonucleotide
SEQ ID No:9	oligonucleotide
SEQ ID No:10	oligonucleotide
SEQ ID No:11	oligonucleotide
SEQ ID No:12	oligonucleotide
SEQ ID No:13	oligonucleotide
SEQ ID No:14	oligonucleotide
SEQ ID No:15	oligonucleotide
SEQ ID No:16	oligonucleotide
SEQ ID No:17	oligonucleotide
SEQ ID No:18	oligonucleotide
SEQ ID No:19	oligonucleotide
SEQ ID No:20	oligonucleotide
SEQ ID No:21	oligonucleotide
SEQ ID No:22	oligonucleotide
SEQ ID No:23	oligonucleotide
SEQ ID No:24	oligonucleotide
SEQ ID No:25	oligonucleotide
SEQ ID No:26	oligonucleotide
SEQ ID No:27	oligonucleotide T73a_U
SEQ ID No:28	oligonucleotide T73a_L

SEQ ID No:29 oligonucleotide minpsb_U
SEQ ID No:30 oligonucleotide minpsb_L
SEQ ID No:31 oligonucleotide
SEQ ID No:32 oligonucleotide
SEQ ID No:33 oligonucleotide
SEQ ID No:34 oligonucleotide
SEQ ID No:35 oligonucleotide
SEQ ID No:36 oligonucleotide
SEQ ID No:37 oligonucleotide ErspU
SEQ ID No:38 oligonucleotide ErspL
SEQ ID No:39 oligonucleotide ErspovL
SEQ ID No:40 oligonucleotide AmboeU

The present invention discloses transgenic plants expressing therapeutically active proteins, in particular antigens. DNA molecules encoding such proteins are expressed from the plastid genome of the plant or are expressed in the cell nucleus and the proteins are targeted to the cell cytosol or to subcellular organelles, such as vacuoles. Plants of the present invention are able to express the proteins in a cost effective manner and are easily accessible. Such plants are able to express large amounts of such proteins in a cost effective manner. Furthermore, therapeutically active proteins expressed in plastids are conveniently packaged, making purification and processing especially easy. Also, such compartmentalization allows the therapeutic molecules to be protected during digestion, thereby favoring oral administration. Therapeutically active proteins expressed in transgenic plants according to the present invention can be administered by a wide range of methods to hosts, including human, pets or livestock, to prevent or treat a variety of diseases, and thereby improve the condition of the treated host. In particular, transgenic plants of the present invention or plant material derived from such plants can be ingested orally by a host and can be used for example to treat allergies, autoimmune diseases or to prevent the rejection of transplantations, preferably by induction of tolerance of the host to antigens. The invention also discloses compositions, such as pharmaceutical compositions, comprising such plants, or plant matter derived from such plants, as well as methods to improve the condition of a host by administration of a composition of the present invention.

Therapeutically Active Proteins Expressed in Transgenic Plants

Proteins of the present invention are preferably able to modulate a host's immune response, examples are given below. Therefore, they can be used to treat or prevent undesired immune responses, for example to suppress or reduce an immune response of the host, for example by inducing tolerance. Alternatively, they can also stimulate the immune system of a host and thereby contribute or result in an immunization of the host against a disease, for example a bacterial, parasitic or viral disease.

In a preferred embodiment, a single protein is expressed in a transgenic plant. In this case, if several proteins are desired for a particular treatment, a mixture of plants each expressing a different protein is used. In an alternate preferred embodiment, several different proteins are expressed in the same transgenic plant. In this case, the DNA molecules encoding the different proteins are included in different expression cassettes which are transformed into the plant or, alternatively, the DNA molecules are included in the same expression cassette. For expression from the plastid genome, for example, the DNA molecules encoding the different therapeutically active proteins can be engineered into an expression cassette to form a single, polycistronic messenger RNA. For simplicity and clarity purposes, "a" therapeutically active protein as mentioned throughout the text refers to as "at least one" therapeutically active protein, meaning one or more proteins. Also, a therapeutically effective amount of a therapeutically active protein of the present invention may be obtained from a single plant or from plant matter derived from a single plant, or may be obtained from a plurality of plants, for examples siblings of the plant.

Plants transformed in accordance with the present invention may be monocots or dicots and include, but are not limited to, maize, wheat, barley, rye, sweet potato, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato, sorghum, sugarcane, sugarbeet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, Arabidopsis, turfgrasses, ornamentals and woody plants such as coniferous and deciduous trees. Once a desired gene has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques.

Also included in the present invention are edible algae, such as unicellular green algae (e.g. Chlamydomonas), multicellular green algae (e.g. *Ulva*), unicellular red algae (e.g.

Porphyridium) and multicellular red algae (e.g. *Porphyra*), which contain plastid genomes substantially similar to those of higher plant that may be transformed in a similar manner.

Expression of Therapeutically Active Proteins in Plant Plastids

The present invention particularly relates to the expression of therapeutically active proteins from the plastid genome of a plant. In this case, some or all of the several thousand copies of the circular plastid genome present in each plant cell are transformed with a DNA molecule encoding a therapeutically active protein of the present invention. The enormous transgene copy number typical of plastids permits expression levels that usually greatly exceed expression levels commonly obtained from nuclear-expressed genes. Such high levels of expression further reduce the cost of production of a therapeutically active protein in plants, and also permits the use of such transgenic plants in applications requiring high levels of the protein in plant material, as for example when the plant or plant material is ingested orally to treat allergies, autoimmune diseases or to prevent the rejection of transplantations. Plastid gene expression also has a number of additional advantages. For example, transgene expression levels are stable over time due to the absence of gene silencing and position effect variation, polycistronic operons can be expressed in a coordinated manner from a single promoter or regulatory sequence allowing the production of equimolar amounts of several proteins, uniparental plastid gene inheritance prevents pollen transmission of foreign DNA in most economically important crops and hence reduces the possibility of lateral transfer to wild or cultivated plants. Also, plastid transgene integration occurs via a homologous recombination process, meaning that precise targeted engineering and gene replacement is readily performed. Furthermore, proteins expressed within the plastid remain sequestered in the organelle and thus are prevented from interacting with the cytoplasmic environment. This feature is essential if the protein expressed in plants also shows activity against or interacts adversely with some components of the plant cytosol.

Plastid transformation technology is described extensively in U.S. Patent Nos. 5,451,513, 5,545,817, 5,545,818 and 5,576,198; in PCT application nos. WO 95/16783 and WO 97/32977; and in McBride et al., Proc. Natl. Acad. Sci. USA 91: 7301-7305 (1994), all of which are incorporated herein by reference. Plastid transformation via biolistics was achieved initially in the unicellular green alga *Chlamydomonas reinhardtii* (Boynton et al. (1988) *Science* 240: 1534–1537, incorporated herein by reference) and this approach,

using selection for *cis*-acting antibiotic resistance loci (spectinomycin/streptomycin resistance) or complementation of non-photosynthetic mutant phenotypes, was soon extended to *Nicotiana tabacum* (Svab et al. (1990) Proc. Natl. Acad. Sci. USA. 87: 8526–8530, incorporated herein by reference).

The basic technique for tobacco plastid transformation involves the particle bombardment of leaf or callus tissue or PEG-mediated uptake of plasmid DNA in protoplasts with regions of cloned plastid DNA flanking a selectable antibiotic resistance marker. The 0.5 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the 156 kb tobacco plastid genome. Initially, point mutations in the chloroplast 16S rDNA and *rps12* genes conferring resistance to spectinomycin and/or streptomycin were utilized as selectable markers for transformation (Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990) Proc. Natl. Acad. Sci. USA 87, 8526-8530; Staub, J. M., and Maliga, P. (1992) Plant Cell 4, 39-45, incorporated herein by reference). This resulted in stable homoplasmic transformants at a frequency of approximately one per 100 bombardments of target leaves. The presence of cloning sites between these markers allowed creation of a plastid targeting vector for introduction of foreign genes (Staub, J.M., and Maliga, P., EMBO J. 12: 601-606 (1993), incorporated herein by reference). Substantial increases in transformation frequency were obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial *aadA* gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3'-adenyltransferase (Svab, Z., and Maliga, P. (1993) Proc. Natl. Acad. Sci. USA 90, 913-917, incorporated herein by reference). Previously, this marker had been used successfully for high-frequency transformation of the plastid genome of the green alga *Chlamydomonas reinhardtii* (Goldschmidt-Clermont, M. (1991) Nucl. Acids Res. 19, 4083-4089, incorporated herein by reference). Recently, plastid transformation of protoplasts from tobacco and the moss *Physcomitrella patens* has been attained using polyethylene glycol (PEG) mediated DNA uptake (O'Neill et al. (1993) *Plant J.* 3: 729-738; Koop et al. (1996) *Planta* 199: 193-201, both of which are incorporated herein by reference). Both particle bombardment and protoplast transformation are appropriate in the context of the present invention.

A DNA molecule encoding a therapeutically active protein of the present invention is inserted into a plastid expression cassette comprising a promoter capable of expressing the DNA molecule in plant plastids. A preferred promoter capable of expression in a plant

plastid is a promoter isolated from the 5' flanking region upstream of the coding region of a plastid gene, which may come from the same or a different species, and the native product of which is typically found in a majority of plastid types including those present in non-green tissues. Gene expression in plastids differs from nuclear gene expression and is related to gene expression in prokaryotes (described in Stern et al. (1997) *Trends in Plant Sciences* 2: 308-315, incorporated herein by reference). Plastid promoters generally contain the -35 and -10 elements typical of prokaryotic promoters and some plastid promoters are recognized by a *E. coli*-like RNA polymerase mostly encoded in the plastid genome and are called PEP (plastid-encoded RNA polymerase) promoters while other plastid promoters are recognized by a nuclear-encoded RNA polymerase (NEP promoters). Both types of plastid promoters are suitable for the present invention. Examples of plastid promoters are promoters of *clpP* genes, such as the tobacco *clpP* gene promoter (WO 97/06250, incorporated herein by reference) and the Arabidopsis *clpP* gene promoter (comprised between positions 71882 and 72,371 in the Arabidopsis plastid genome, the sequence of which was made available by Takakazu Kaneko and Satoshi Tabata of the Kazusa DNA Research Institute at the URL: ftp://genome-ftp.stanford.edu/pub/arabidopsis/chloroplast/
). Another promoter that is capable of expressing a DNA molecule in plant plastids comes from the regulatory region of the plastid 16S ribosomal RNA operon (Harris et al., *Microbiol. Rev.* 58:700-754 (1994), Shinozaki et al., *EMBO J.* 5:2043-2049 (1986), both of which are incorporated herein by reference). Other examples of promoters that are capable of expressing a DNA molecule in plant plastids are a *psbA* promoter or a *rbcL* promoter. A plastid expression cassette also preferably further comprises a plastid gene 3' untranslated sequence (3' UTR) operatively linked to a DNA molecule of the present invention. The role of untranslated sequences is preferably to direct the 3' processing of the transcribed RNA rather than termination of transcription. Preferably, the 3' UTR is a plastid *rps*16 gene 3' untranslated sequence or the Arabidopsis plastid *psbA* gene 3' untranslated sequence. In a further preferred embodiment, a plastid expression cassette comprises a poly-G tract instead of a 3' untranslated sequence. A plastid expression cassette also preferably further comprises a 5' untranslated sequence (5' UTR) functional in plant plastids operatively linked to a DNA molecule of the present invention.

A plastid expression cassette is comprised in a plastid transformation vector, which preferably further comprises flanking regions for integration into the plastid genome by homologous recombination. The plastid transformation vector may optionally comprise at

least one chloroplast origin of replication. The present invention also encompasses a plant plastid transformed with such a plastid transformation vector, wherein the DNA molecule is expressible in the plant plastid. The invention also encompasses a plant or plant cell, including the progeny thereof, comprising this plant plastid. In a preferred embodiment, the plant or plant cell, including the progeny thereof, is homoplasmic for transgenic plastids. Other promoters that are capable of expressing a DNA molecule in plant plastids are transactivator-regulated promoters, preferably heterologous with respect to the plant or to the subcellular organelle or component of the plant cell in which expression is effected. In these cases, the DNA molecule encoding the transactivator is inserted into an appropriate nuclear expression cassette which is transformed into the plant nuclear DNA. The transactivator is targeted to plastids using a plastid transit peptide. The transactivator and the transactivator-driven DNA molecule are brought together either by crossing to a selected plastid-transformed line a transgenic line containing a DNA molecule encoding the transactivator supplemented with a plastid-targeting sequence and operably linked to a nuclear promoter, or by directly transforming a plastid transformation vector containing the desired DNA molecule into a transgenic line containing a DNA molecule encoding the transactivator supplemented with a plastid-targeting sequence and operably linked to a nuclear promoter. If the nuclear promoter is an inducible promoter, in particular a chemically inducible promoter, expression of the DNA molecule in the plastids of plants is activated by foliar application of a chemical inducer. Such inducible transactivator-mediated plastid expression system is preferably tightly regulatable, with no detectable expression prior to induction and exceptionally high expression and accumulation of protein following induction. A preferred transactivator is for example viral RNA polymerase. Preferred promoters of this type are promoters recognized by a single sub-unit RNA polymerase, such as the T7 gene 10 promoter, which is recognized by the bacteriophage T7 DNA-dependent RNA polymerase. The gene encoding the T7 polymerase is preferably transformed into the nuclear genome and the T7 polymerase is targeted to the plastids using a plastid transit peptide. Promoters suitable for nuclear expression of a gene, for example a gene encoding a viral RNA polymerase such as the T7 polymerase, are described *infra*. Expression of the DNA molecules in plastids can be constitutive or can be inducible Expression of the DNA molecules in the plastids can be also organ- or tissue-specific. These different embodiment are extensively described in WO 98/11235, incorporated herein by reference.

Nuclear Expression of a Transactivator or of Therapeutically Active Proteins

For expression in a particular transgenic plant, a nucleotide sequence according to the invention may require modification and optimization. Low expression in transgenic plants may result from heterologous nucleotide sequences having codons which are not preferred in plants. It is known in the art that all organisms have specific preferences for codon usage, and the codons of the nucleotide sequence described in this invention can be changed to conform with plant preferences, while maintaining the amino acids encoded thereby. Furthermore, high expression in the plant nucleus is best achieved from coding sequences which have at least 35% GC content, and preferably more than 45%. Additionally, nucleotide sequences which have low GC contents may express poorly in plant nuclei due to the existence of ATTTA motifs which may destabilize messages, and AATAAA motifs which may cause inappropriate polyadenylation. Although preferred gene sequences may be adequately expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al.* Nucl. Acids Res. 17: 477-498 (1989)). Also, the DNA molecule is screened for the existence of illegitimate splice sites which cause message truncation. All changes required to be made within the DNA molecule such as those described above are made using well known techniques of e.g. site directed mutagenesis, PCR, and synthetic gene construction using the methods described in the published patent applications EP 0 385 962, EP 0 359 472, and WO 93/07278.

For efficient initiation of translation, sequences adjacent to the initiating methionine may require modification. For example, sequences known to be effective in plants can be included. Joshi has reported an appropriate consensus sequence for plant nuclei (NAR 15: 6643-6653 (1987)) and Clontech suggests a further consensus translation initiator (1993/1994 catalog, page 210). These consensus sequences are suitable for use with DNA molecules of this invention. The sequences are incorporated into constructions comprising the DNA molecules, up to and including the ATG (whilst leaving the second amino acid unmodified), or alternatively up to and including the GTC subsequent to the ATG (with the possibility of modifying the second amino acid of the transgene).

Expression of a nucleotide sequence encoding a transactivator or a therapeutically active protein in transgenic plants is driven by a promoter shown to be functional in plants. The choice of promoter depends on the temporal and spatial requirements for expression, and

also depending on the host species. Expression of a DNA molecule of the present invention in plants can be constitutive or inducible, for example chemically inducible, or the expression of the DNA molecule can be organ- or tissue-specific. Although many promoters from dicotyledons have been shown to be operational in monocotyledons and *vice versa*, ideally dicotyledonous promoters are selected for expression in dicotyledons, and monocotyledonous promoters for expression in monocotyledons. However, there is no restriction to the provenance of selected promoters; it is sufficient that they are operational in driving the expression of the nucleotide sequence in the desired cell. Preferred promoters which are expressed constitutively include the CaMV 35S and 19S promoters, and promoters from genes encoding actin or ubiquitin. A DNA molecule of this invention can also be expressed under the regulation of promoters which are chemically regulated. Preferred technology for chemical induction of gene expression is detailed in patent application EP 0 332 104 and US patent 5,614,395. A preferred promoter for chemical induction is the tobacco PR-1a promoter. A preferred category of promoters is that which is wound inducible. Numerous promoters have been described which are expressed at wound sites and also at the sites of phytopathogen infection. Ideally, such a promoter is only active locally at the sites of infection. Preferred promoters of this kind include those described by Stanford *et al.* Mol. Gen. Genet. 215: 200-208 (1989), Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), and Warner *et al.* Plant J. 3: 191-201 (1993). Preferred tissue specific expression patterns include green tissue specific, root specific, stem specific, fruit specific and flower specific. Promoters suitable for expression in green tissue include many which regulate genes involved in photosynthesis, and many of these have been cloned from both monocotyledons and dicotyledons. A preferred promoter is the maize PEPC promoter from the phosphoenol carboxylase gene (Hudspeth & Grula, Plant Molec. Biol. 12: 579-589 (1989)). A preferred promoter for root specific expression is that described by de Framond (FEBS 290: 103-106 (1991); EP 0 452 269) and a further preferred root-specific promoter is that from the T-1 gene (de Framond et al. FEBS 290: 103-106 (1991); EP 0 452 269). A preferred stem specific promoter is that described in US patent 5,625,136 and which drives expression of the maize *trpA* gene. Preferred embodiments of the invention are transgenic plants expressing a DNA molecule in a root-specific fashion. Further preferred embodiments are transgenic plants expressing a DNA molecule in a wound-inducible or

pathogen infection-inducible manner. Additional promoters are synthetic promoters such as the Gelvin Super MAS promoter (Ni et al. (1995) Plant J. 7: 661-676).
In addition to the selection of a suitable promoter, constructions for expression of a DNA molecule in the plant nucleus preferably comprise appropriate 3' sequences, such as 3' regulatory sequences or transcription terminators, to be operably linked downstream of the heterologous nucleotide sequence. Several such terminators are available and known in the art (*e.g. tm1* from CaMV, E9 from *rbcS*). Any available terminator known to function in plants can be used in the context of this invention. Numerous other sequences can be incorporated into expression cassettes for a DNA molecule described in this invention. These include sequences which have been shown to enhance expression such as intron sequences (*e.g.* from *Adh1* and *bronze1*) and viral leader sequences (*e.g.* from TMV, MCMV and AMV).
In another preferred embodiment, the nuclear expressed DNA molecule of the present invention is targeted to a subcellular location or locations in the plant. For example, a therapeutically active protein is secreted from the cell into the apoplast or a therapeutically active protein is targeted to a particular subcellular organelle, for example to the vacuoles or to the endoplasmic reticulum. This is achieved for example by the fusion of the appropriate targeting sequences to a DNA molecule of the present invention using techniques well known in the art. Thus for targeting to the apoplast or to the vacuole, the protein preferably comprises an appropriate signal peptide, preferably at its N-terminus, which allows targeting of the protein to the organelle. In a preferred embodiment, a protein of the present invention is targeted to the vacuoles. For targeting of the protein to the vacuoles, in addition to an N-terminal signal peptide, the protein preferably also further comprises a vacuolar targeting sequence, such as that from a tobacco chitinase gene, preferably at its C-terminus (Neuhaus et al. (1991) Proc. Natl. Acad. Sci. USA 88, 10362-10366). The expression of a protein encoded by a DNA molecule of the present invention can also be targeted to the plastids using an appropriate plastid transit peptide, preferably comprised at the N-terminus of the protein as described in details *infra*. A protein of the present invention can also be targeted to the mitochondrion, for example by fusion with a mitochondrial targeting sequence, such as a *N. plumbaginifolia* F1-ATPase β-subunit (Chaumont et al. (1994) Plant Molecular Biology 24: 631-641).
Vectors suitable for plant transformation are described elsewhere in this specification. For *Agrobacterium*-mediated transformation, binary vectors or vectors carrying at least one T-

DNA border sequence are suitable, whereas for direct gene transfer any vector is suitable and linear DNA containing only the construction of interest may be preferred. In the case of direct gene transfer, transformation with a single DNA species or co-transformation can be used (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)). For both direct gene transfer and *Agrobacterium*-mediated transfer, transformation is usually (but not necessarily) undertaken with a selectable marker which may provide resistance to an antibiotic (e.g. kanamycin, hygromycin or methotrexate) or a herbicide (e.g. Basta/phosphinothricin or an inhibitor of protoporphyrinogen oxidase). The choice of selectable marker is not, however, critical to the invention. Examples of expression cassettes and transformation vectors are described in further detail *infra*.

The DNA molecules of the present invention are introduced into the plant cell in a number of well known ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway et al., BioTechniques 4:320-334 (1986)), electroporation (Riggs and Bates, Proc. Natl. Acad. Sci. USA 83:5602-5606 (1986)), *Agrobacterium*-mediated transformation (Hinchee et al., Biotechnology 6:915-921 (1988)), direct gene transfer (Paszkowski et al., EMBO J. 3:2717-2722 (1984)), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (see, for example, U.S. Patent 4,945,050; and McCabe et al., Biotechnology 6:923-926 (1988); *see also* Weissinger et al., Annual Rev. Genet. 22:421-477 (1988); Sanford et al., Particulate Science and Technology 5:27-37 (1987)(onion); Christou et al., Plant Physiol. 87:671-674 (1988) (soybean); McCabe et al., Bio/Technology 6:923-926 (1988)(soybean); Datta et al., Bio/Technology 8:736-740 (1990)(rice); Klein et al., Proc. Natl. Acad. Sci. USA, 85:4305-4309 (1988)(maize); Klein et al., Bio/Technology 6:559-563 (1988)(maize); Klein et al., Plant Physiol. 91:440-444 (1988)(maize); Fromm et al., Bio/Technology 8:833-839 (1990); and Gordon-Kamm et al., Plant Cell 2:603-618 (1990)(maize); Koziel *et al.* (Biotechnology 11: 194-200 (1993))(maize); Shimamoto *et al.* Nature 338: 274-277 (1989)(rice); Christou *et al.* Biotechnology 9: 957-962 (1991)(rice); European Patent Application EP 0 332 581 (orchardgrass and other *Pooideae*); Vasil *et al.* (Biotechnology 11: 1553-1558 (1993)(wheat); Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993) (wheat); Wan *et al.* (Plant Physiol. 104: 37-48 (1994)(barley)); Umbeck *et al.*, (Bio/Technology 5: 263-266 (1987)(cotton).

Administration of Therapeutically Active Proteins to a Host
An advantage of the present invention is the wide variety of ways by which a therapeutically active protein expressed in transgenic plants can be administered to a host such as, for example, orally, enterally, nasally, parenterally, particularly intramuscularly or intravenously, rectally, topically, ocularly, pulmonarilly or by contact application. In a preferred embodiment, an allergen expressed in transgenic plants is administered to a host orally.
In a preferred embodiment, a therapeutically active protein expressed in a transgenic plant is extracted and purified, and used for the preparation of a pharmaceutical composition. Localization of the expressed therapeutically active protein in the plastids greatly facilitates such extraction and purification. For example, intact plastids are first isolated by centrifugation making the extraction and purification of the therapeutically active protein easier. In another preferred embodiment, proteins are isolated and purified in accordance with conventional conditions and techniques known in the art previously used to isolate such proteins, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like. Such compositions typically comprise an effective amount of a therapeutically active protein together with one or more organic or inorganic, liquid or solid, pharmaceutically suitable carrier materials. A therapeutically active protein produced according to the present invention is employed in dosage forms such as tablets, troches, dispersions, suspensions, solutions, capsules, creams, ointments, aerosols, powder packets, or liquid solutions as long as the biological activity of the protein is not destroyed by such dosage form. For example, the protein may be provided as a pharmaceutical composition by means of conventional mixing, granulating, dragee-making, dissolving, lyophilizing or similar processes. The dosage of the protein is dependent upon the weight, age, and physical and pharmacokinetical condition of the patient and is further dependent upon the method of delivery.
Where the protein is administered enterally, it may be introduced in solid, semi-solid, suspension or emulsion form and may be compounded with any pharmaceutically acceptable carriers, including water, suspending agents, emulsifying agents. The proteins of the invention may also be administered by means of pumps, or in sustained-release form, especially, when administered as a preventative measure, so as to prevent the development of disease in a subject or when administered to ameliorate or delay an already established disease.

Therapeutically active proteins produced according to the present invention are particularly well-suited for oral administration as pharmaceutical compositions. Compositions for oral administration include proteins provided as dry powders, food-stuffs, aqueous or non-aqueous solvents, suspensions or emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous carriers include water, water-alcohol solutions, emulsions or suspensions, including saline and buffered medical parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, Ringer's solution containing lactose, or fixed oils. In a preferred embodiment, such compositions are ingested orally alone or ingested together with food or feed or a beverage.
In another preferred embodiment, a transgenic plant expressing a therapeutically active protein of the present invention or plant matter derived from such plant are administered orally as medical food. Such edible compositions are consumed by eating if in a solid form or by drinking if in a liquid form. In a preferred embodiment, the transgenic plant material is directly ingested without a prior processing step or after minimal culinary preparation. For example, the therapeutically active protein is expressed in a plant parts of which can be eaten directly, such as a fruit or a vegetable. Preferably, the protein is expressed in the plastids of a plant part which can be eaten. All types of plastids in any plant are suitable for the present invention. For example, the protein is expressed in spinach or lettuce chloroplasts, in tomato chromoplasts or in potato amyloplasts. In an alternative preferred embodiment, the plant is processed and the plant material recovered after the processing step is ingested. Processing methods preferably used in the present invention are methods commonly used in the food or feed industry. The final products of such methods still comprise a substantial amount of the protein and can be conveniently eaten or drunk. The final product may also be mixed with other food or feed forms, such as salts, carriers, flavor enhancers, antibiotics and the like, and consumed in solid, semi-solid, suspension, or emulsion form. In another preferred embodiment, such methods comprise a conservation step, such as, e.g., pasteurization, cooking or addition of conservation and preservation agents. Any plant is used and processed in the present invention to produce edible or drinkable plant matter. When tobacco plants are used according to the present invention, for example, it may be necessary to treat the plants or plant material to remove harmful substances such as nicotine. In a preferred embodiment, a low-alkaloid tobacco plant is used for the expression of a protein of the present invention. In a preferred embodiment, the amount of therapeutically active protein in an edible or drinkable plant matter according

to the present invention is tested. Such amount is determined e.g. by Elisa or Western blot analysis using an antibody specific for the protein. This determination is used to standardize the amount of protein ingested. For example the amount of therapeutically active protein in a juice, e.g. in a tomato juice, is determined and regulated, for example by mixing batches of product having different levels of protein, so that the quantity of juice to be drunk per dose can be standardized. It is clear that the present invention provides novel and efficient ways to produce and administer plant-expressed therapeutically active agents as medical foods.

A therapeutically active protein produced in a plant and eaten by the host is absorbed by the digestive system. One advantage of the ingestion of a plant or plant material, particularly intact plants or plant material, or plant material which has been only mildly processed, is to provide encapsulation or sequestration of the protein in the cells of the plant. Thus, the protein may receive at least some protection from digestion in the upper digestive tract before reaching the gut or intestine and a higher proportion of active protein would be thus available for uptake.

When a plant used for expression of a therapeutically active protein of the present invention is tobacco, the crude, so-called “F2 soluble protein fraction” can for example be used for therapeutical administration. An additional fraction, F1, is produced by precipitation of high molecular weight protein complexes from a crude tobacco extract. In non-transgenic plants, this F1 fraction consists almost exclusively of ribulose bisphosphate carboxylase as disclosed in US patent 4,347,324. It is possible that some transgenic therapeutically active proteins which are produced by the methods of the present invention may partition into the F1 fraction as well. In this case, the F1 fraction could also be used for therapeutical administration.

Preferred hosts or recipients for therapeutically active proteins of the present invention are animals. Animals preferably include vertebrates, preferably mammals. Preferred mammals are humans, pets or companion animals, like e.g. cats, dogs, rodents, ferrets, primates, fishes or birds, or farm animals, like cows, hogs, poultry, or the like.

Immunization

Several current vaccines need expensive refrigeration and are therefore not readily available in developing countries. Also, efficient vaccines against some pathogens have still not been produced in large enough quantities or are still not sufficiently safe for broad distribution. In an attempt to improve the situation, a hepatitis B viral surface antigen has

been expressed in plants and administered through edible plants with the goal of vaccinating against hepatitis B (see for example US patents 5,484,719 and 5,612,487). However, the genes encoding the vaccines were expressed from the plant nuclear genome and only relatively low yields of the vaccines could obtained, thus precluding the efficient production of an edible vaccine. Immunization against pathogenic microorganisms has also been attempted by expressing antigenic determinants of the pathogen in transgenic plants and administering the plant orally to a host (US patents 5,654,184, 5,679,880, 5,686,079). However, in these cases too, relatively low levels of expression of the transgene form the plant nuclear genome have limited the commercial success of such approaches.
It is therefore a preferred embodiment of the present invention to express antigens capable of inducing an immunization of a host against a pathogen, for example a bacterial, a parasitic or a viral pathogen or to immunize a host against a toxin, in transgenic plants, in particular in subcellular organelles, preferably in vacuoles, more preferably in plant plastids. Plants of the present invention are used for the immunization of humans against for example poliomyelitis, measles, mumps, rubella, smallpox, yellow fever, viral hepatitis B, influenza, rabies, adenoviral infections, Japanese B encephalitis, varicella, diarrhea, acute respiratory infections, malaria, pertussis, diphteria, tetanus or neonatal tetanus. Such proteins are also advantageously used for the immunization of animals to prevent diseases such as for example equine infections, canine distemper, rabies, canine hepatitis, parvovirus, and feline leukemia, Newcastle, Rinderpest, hog cholera, blue tongue and foot-mouth, brucellosis, fowl cholera, anthrax and black leg, as well as diseases resulting from infections with protozoans and helminths. Plants of the present invention are also used for the immunization of animals, including humans, against various toxins or irritants, such as snake or bee venom, mosquito saliva, poison ivy. The host becomes immunized upon administration of the antigen, preferably by oral ingestion of the plant or of plant matter derived from the plant. An adjuvant, as well-known in the immunological art, may be added to an composition comprising an antigen of the present invention in an amount such as to improve the immunological activity of the composition and thereby the therapeutical effectiveness of the composition. Information relating to the immune system and to immunological responses are also found in Hood et al. (1984) Immunology. The Benjamin/Cummings Publishing Co, Inc. Menlo Park, CA.

Tolerization

The field of the medical arts relating to the suppression or reduction of undesired immune responses also needs improvements. An animal's immune system is an intricate network of specialized cells and organs acting upon various specific signals. One of its major functions is to discriminate between "self" and "non-self". This basic property allows for protection of the host against invading pathogens without provoking detrimental immune responses against the host itself. Typically, an immune response is characterized by a cellular response provided by certain cells of the lymphoid system and a humoral response provided by antibodies, and is initiated by the encounter of a non-self determinant by the immune system resulting in the destruction of the non-self. However, in some cases, the discrimination between self and non-self becomes deficient, resulting in an immunological response against some parts of the self and leading to the development of an autoimmune disease. In some other cases, a immunological response against specific non-self antigens results in undesired effects such as, for example, the rejection of a non-autologous tissue or organ transplant, or the development of an allergic response against an environmental determinant. In these cases, an effective suppression or reduction of the immune response is desirable. Several strategies have been attempted to reach this goal. For example, certain autoimmune diseases have been prevented by aerosol administration of autoantigens (US patents 5,641,473 and 5,641,474). Other treatment attempts have been based on oral tolerization of the host to an antigen, a mechanism by which ingested proteins cause a suppression or reduction of the usual immune response to specific foreign substances or autoantigens (see for example Weiner (1994) Proc. Natl. Acad. Sci. 91, 10,762-10,765; Friedman et al. (1994) in Grandstein RD (ed): Mechanisms of Immune Regulation, Chem. Immunol. Basel, Karger, Vol 58, 259-290). There are multiple mechanism involved in oral tolerance, two of the primary ones being active cellular suppression or clonal anergy. This is thought to be associated with absorption by mucosa of the small intestine. Antigens are taken up in the gut and presented to specialized mucosal tissues, such as Peyer's patch cells, which are the point of entry to the gut-associated immune system. This induced immunological hypo- or unresponsiveness is related to the suppression of food allergy that occurs normally in most individuals and is the primary means of tolerization to the numerous potential antigens which enter the digestive tract as components of the diet. In the therapeutical area, oral administration of insulin has provided some relief in the treatment of type I diabetes (US patent 5,763,396) and oral administration of type I or type III collagen had some success in the treatment of autoimmune arthritis (US patent 5,733,547). Additionally, attempts to suppress the rejection of a transplant by oral

administration of polymorphic class II MHC allopeptides has been made (US patent 5,593,698). In these previous cases, the antigen was purified from natural sources. However, for an oral tolerization to be effective, high amount of antigens have to be ingested. Such high amounts are difficult or expensive to obtain for many antigens, in particular for airborne antigens, and cannot be easily prepared in an orally ingestible composition. Thus, the success of many of such strategies has been hampered by the lack of sufficient amounts of protein available for oral ingestion. Recently, the expression in transgenic plants of certain transplantation antigens and autoantigens and their enteral or oral administration has been attempted (WO 95/08347). However, here again relatively low levels of nuclear transgene expression are likely to preclude a successful outcome of these approaches.

Therefore, in a preferred embodiment, antigens capable of suppressing or reducing an immune response or an inflammatory condition of a host are expressed in plants, in particular in plant plastids. Preferably such antigens are administered orally to the host. Transgenic plants expressing such antigens are particularly beneficial in the treatment, prevention or amelioration of diseases such as for example allergies, autoimmune diseases or rejection of transplantations, preferably by inducing tolerance of the host to one or multiple antigens. For oral ingestion, the plant expressing the antigen or plant matter derived from such plants is either eaten raw or eaten or drunk after a processing step, for example a processing step described *supra*. In a preferred embodiment, the result of the oral ingestion of the plant or plant matter is an induction of tolerance of the host against the antigen. The present invention for the first time provides for a plentiful and cheap source of antigens which can be used for oral tolerization by expressing such antigens from the plastid genome of a plant. In a preferred embodiment, edible plants engineered to overexpress specific peptide antigens as described in the present invention are an ideal vehicle for an oral tolerization therapy based on medical food. The use of transgenic plants for oral tolerance has a number of potential advantages over current approaches (biochemical purification or recombinant expression in cell culture systems and protein expression in transgenic milk). These advantages include low production cost per dose, minimal risk of transmitting mammalian pathogens, no or limited need for purification, processing or encapsulation when antigen-containing plants or plant material are consumed directly as food.

In another preferred embodiment, an antigen of the present invention is co-administered to a host with a tolerizing adjuvant. Such an adjuvant is for example a toxin, such as a cholera toxin B subunit (as described for example in Arakawa et al. (1998) *Nat Biotechnol* 16: 934-8). Such toxin subunit molecules are known in the art to promote oral tolerance as opposed to mucosal immunity (Sun et al PNAS (1996) 93:7196-201). Another tolerizing adjuvant used in the present invention is an *E. coli* labile enterotoxin B. In a preferred embodiment, a nucleic acid sequence encoding the toxin is fused to a nucleic acid sequence encoding the antigen to produce a DNA molecule encoding a fusion protein. A plant comprising such DNA molecule is produced as described in the present invention and the fusion protein is administered to the host as described in the present invention. In another preferred embodiment, the toxin and the antigen are expressed in a plant as separate polypeptides and administered to the host as described in the present invention. Alternatively, the toxin and the antigen are expressed in different plants and combined prior to administration to the host. Alternatively, the toxin is obtained from a different organism, preferably purified, and then combined with the antigen prior to administration to the host.

Allergens

Allergies cause a major discomfort in a large proportion of the population and are suspected to also have adverse effects on animals. The range of magnitude of allergic reactions in a human population ranges from mild symptoms to heavy and dramatic appearances, such as anaphylactic shocks, and common pathological appearances include food allergies, skin reactions (urticaria or atopic dermatitis), allergic reactions of the upper respiratory tract (e.g. hayfever or allergic rhinitis) or allergic reactions of the lower respiratory tract that are a major cause of asthma (for a review, see O'Hehir et al. (1991) Annu. Rev. Immunol. 9: 67-95). Allergic responses are typically due to exaggerated IgE responses to various types of allergens (for example pollen, animal dander, insect fecal matter or dust as well as food allergens) and are often associated with inflammatory responses. The pathological manifestations of such IgE-antigen interactions are due in particular to mast-cell degranulation that results in the release of histamine, heparin and leukotrienes. Present medications are mainly unspecific and are in the form of a treatment with e.g. antihistamines or epinephrine, an antagonist of mast-cell degranulation. More specific treatments have also been developed and are based on periodic injections of suballergic doses of the allergen, resulting in a desensitization of the host against the allergen.

However, such injections are cumbersome and desensitization addresses barely or not at all the T cell response usually associated with allergies. Injections for desensitization are therefore often of limited efficacy. The present invention provides novel, specific methods for the treatment of allergies by expressing DNA molecules encoding allergens in plants and administering such plants or plant matter derived from such plants to a host. Such plants or plant matter are preferably administered orally to the host who thereby develops tolerance to the allergen. Treatment of infants or young individuals whose pedigree suggests that they may be at risk for the onset of allergic diseases later in life may also be undertaken in a prophylactic manner to prevent the development of allergy. Alternatively, allergens expressed in transgenic plants of the present invention are extracted and purified according to methods well-known in the art and used for desensitization of a host against the allergen by periodic injections of suballergic doses of the allergen.
Suitable allergens for use in the context of the present invention includes food allergens, drug allergens, venom allergens, plant allergens, fungal allergens, bacterial allergens, animal allergens, other allergens from naturally-occurring or synthetic substances and extracts thereof. Food allergens include, for example, seafood, strawberries, fresh fruit and vegetables, peanuts and cow's milk. Drug allergens include, for example, penicillin and insulin. Venom allergens include, for example, bee, wasp and mosquito venoms. A preferred allergen is bee venom peptide PLA-2. Plant allergens include, for example allergens from pollen, such as, tree pollen, grass pollen and weed pollen. Fungal allergens include, for example, mold spores. Animal allergens include, for example, dander or saliva from dogs, cats, horses, etc. Preferred allergens of the present invention are house dust mite allergens, such as allergens from *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, preferably the *Der f I, Der f II, Der p I and Der p II* allergens (US patents 5,552,142, 5,770,202 and 5,798,099). Alternative preferred allergens are derived from ryegrass pollen, e.g from *Lolium perenne* and include isolated peptides of *Lol p V* (US patent 5,710,126). Further alternate preferred allergens include allergens derived from Johnson grass pollen, such as *Sor h I*, a major allergen derived from *Sorghum halepense* (US patents 5,480,972 and 5,691,167). Further preferred allergens include ragweed pollen allergen, such as the *Amb a I* and *Amb a II* allergens (US patent 5,698,204). Further preferred allergens also include the *Aln g I* allergen of alder, *Alnus* sp., the *Cor a I* allergen of hazel (*Corylus* sp.) and the *Bet v I* allergen of birch (*Betula* sp.), described in US patent 5,693,495. Further allergens include cat antigens, such as the *Fel d I* allergen (US patent

5,328,991), and dog dander allergens, such as *Can f II* (US patent 5,939,283). Further allergen also include the *rAed a 1* and *rAed a 2* allergens from the mosquito *Aedes aegypty* (WO 98/04274) and antigens of the honey bee venom (Lomnitzer and Rabson (1986) J. Allergy Clin. Immunol. 78: 25-30) or murine urinary proteins (Gurka et al. (1989) J. Allergy Clin. Immunol. 83: 945-954).
Furthermore, it has been suggested that the anaphylactic reaction and the IgE-mediated reaction are separable and that disulfide bridges are largely responsible for anaphylaxis. The removal of cystein residues involved in forming disulfide bridges in *Dermatophagoides farinae Der f II* allergen has been shown to greatly reduce the anaphylactic reaction without altering the allergenic epitopes (Takai et al. (1997) Nature Biotechnology 15: 754-758). The present invention also encompasses the expression of allergens modified in this manner so as to lack disulfide bridges. A DNA molecule encoding an allergen is cloned in a plastid transformation vector using techniques well-know in the art and transgenic plants are produced. Plants or plant matter of the present invention are administered to a host alone or in combination with other therapies against allergies.

Antigens Expressed in Transgenic Plants

It is a preferred embodiment of the present invention to express antigens in transgenic plants, in particular in plant plastids. Preferably, such antigens are capable of modulating the immune system of a host in need thereof, resulting in the desired therapeutic effect. Transgenic plants expressing such antigens are particularly beneficial in the treatment, prevention or amelioration of diseases such as for example allergies, autoimmune diseases or rejection of transplantations, preferably by inducing tolerance of the host to one or multiple antigens, or in immunization procedures. Such transgenic plants are administered to a host by different methods known in the art, preferably orally, preferably by eating or drinking a plant of the present invention or plant matter derived from such plants.

Antigens in Autoimmune Diseases or Self-Antigens

An autoimmune disease is a malfunction of the immune system of an animal, including a human, in which the immune system fails to distinguish between foreign substances within the animal and substances which are part of the animal's normal composition. As a failure of immunological tolerance, T cells or B cells, or both, emerge bearing receptors allowing them to recognize and attack self components. This results in an autoimmune disease in

the host bearing such T cells or B cells. Numerous diseases have been diagnosed to be caused at least in part by autoantigens. For example, blood cells are the most common type of cells affected, leading to diseases such as thrombocytopenic purpura, in which antibodies against platelets are formed, or agranulocytosis, where autoantibodies are formed against polymorphonuclear leukocytes. Hemolysis and anemia has also been reported to be due in some cases to autoantibodies directed at the surface of erythrocytes. Antibodies to cell-surface receptors can also cause diseases by interfering with receptor function, such as in the case of myasthenia gravis, where antibodies are formed against acetylcholine receptors, thus impeding neuromuscular transmission. The opposite effect, the stimulation of a receptor by an anti-receptor autoantibody has also been discovered in Grave's disease or hyperthyroidism. Further examples of diseases where a failure of immunological tolerance is suspected or known include for example multiple sclerosis, diabetes mellitus, systemic lupus erythematosus, polychondritis, systemic sclerodoma, Wegener's granulamatosis, dermatomyositis, chronic active hepatitis, psoriasis, Steven-Johnson syndrome, idiopathic sprue, autoimmune inflammatory bowel disease (including e.g. ulcerative colitis and Crohn's disease), sarcoidosis, primary biliary cirrhosis, uveitis (anterior and posterior), keraconjunctivitis sicca and vernal keraconjunctivitis, interstitial lung fibrosis, psoriatic arthritis and glomerulonephritis (with and without nephrotic syndrome, e.g. including idiopathic nephrotic syndrome or minimal change nephropathy), and arthritis (for example rheumatoid arthritis, arthritis chronica progrediente and arthritis deformans).
For autoimmune diseases as for allergies, present medications largely lack specificity and are often connected with unpleasant side effects, such as global immunosuppression of the host. It is therefore a preferred embodiment of the present invention to provide for novel treatments for autoimmune diseases by expressing self-antigens that are targeted by autoantibodies in plant plastids. Such plants or plant matter derived from such plants is administered to a host in need thereof, preferably orally, preferably by being eaten or drunken by the host. Tolerance of the host to the specific autoantibodies then develops without affecting the general immune response abilities of the host. Plants or plant matter of the present invention are administered to a host alone or in combination with immunosuppressant or anti-inflammatory agents, including cyclosporins, rapamycins, FK 506 and steroids.
Preferred self-antigens of the present invention are for example collagen, preferably type I or type III collagen, to treat or prevent arthritis, preferably rheumatoid arthritis (see US patent 5,733,547), myelin basic protein to treat or prevent multiple sclerosis, S-antigen to

treat or prevent autoimmune uveoretinitis, insulin to treat or prevent type I diabetes (see US patent 5,763,396), glutamic acid decarboxylase or an islet cell-specific antigen to treat or prevent diabetes, thyroglobulin to treat or prevent autoimmune thyroiditis, acetylcholine receptor to treat or prevent myasthenia gravis.

A DNA molecule encoding an autoantigen is cloned in a plastid transformation vector or into a transformation vector for nuclear transformation using techniques well-know in the art and transgenic plants are produced.

Transplantation Antigens

There is a great need for grafts or transplantations for the replacement of severely injured tissues and organs. Grafts from an individual to himself (autografts) generally succeed but grafts from genetically dissimilar individuals of the same species (allogeneic grafts) or between individuals of different species (xenogeneic grafts) do not normally succeed without immunosuppressive drugs. Without continued immunosuppressive drug therapy, the organ will be rejected. A general method to induce tolerance to the transplanted organ and to improve the chances of success of an allo- or xenogeneic graft has therefore great potential and is addressed in the present invention.

A transplant rejection is initiated by an immune response to the cell-surface antigens that distinguish donor from host. Such cell-surface antigens mainly belong to the histocompatibility antigens, in particular to the major histocompatibility complex (MHC). Products of the class I and II MHC genes are involved in presenting antigens. They are therefore particularly important in the recognition of nonself antigens by T cells and play an important role in transplantation rejection. Class I MHC complexes comprise a transmembrane glycoprotein homodimer (heavy chain, HC) attached to a b2 microglobulin moiety (Bjorkman et al. (1987) Nature 329: 506). Three class Ia loci (HLA-A, B and C in humans, H-2K, H-2D and H-2L in mice) and several class Ib loci encoding the HC moiety have been identified (York and Rock (1996) Annu Rev Immunol 14: 369-396). Class II MHC are heterodimers composed of two glycoproteins, the a and b chains (Brown et al. (1993) Nature 364: 33). Since the MHC loci are conserved among vertebrates, in particular among mammals (Klein (1986) Natural History of the Major Histocompatibility Complex. Oxford: Blackwell Sci.), DNA molecules encoding MHC antigens can also be isolated from various mammals, including pig, a preferred donor for xenotransplantations.

The importance of MHC antigens in transplantation rejection has been further established by showing that targeting of class II molecules by monoclonal antibodies attenuates

transplant rejection. Therefore, MHC antigens are good candidates for a therapy involving tolerization of a graft recipient against specific MHC determinant of a donor. Recently, the expression in transgenic plants of certain transplantation antigens and autoantigens and their enteral or oral administration, has been attempted (WO 95/08347). However, here again relatively low levels of nuclear transgene expression are likely to preclude a successful outcome of these approaches.

In the present invention, MHC genes, in particular a gene encoding a class I HC moiety and class II MHC genes encoding the a or b chain, are expressed from the plastid genome or the nuclear genome of a plant. Transgenic plants comprising such plastids are administered to a host, preferably prior to and/or after a tissue- or organ transplantation (allo- or xeno-transplantation), to induce tolerization of the host. Preferably, such plants or matter derived from such plants is administered orally to the host to induce an oral tolerization of the host. DNA molecules encoding the different known allotypes of MHC class I HC gene and class II MHC genes encoding the a or b chain are introduced in transgenic plants. For use in a particular transplantation, allotypes of both donor and recipient are determined and the recipient is administered, preferably orally, plant matter comprising selected MHC antigens of the donor. Plants or plant matter of the present invention are administered to a host alone or in combination with immunosuppressant, including e.g. cyclosporins.

Other Therapeutically Active Proteins

Other therapeutically active proteins expressed in plants in the context of the present invention are e.g., but not limited to, blood proteins (e.g. clotting factors VIII and IX, complement factors and complements, hemoglobins or other blood proteins, serum albumin, and the like), hormones (e.g. insulin, growth hormone, thyroid hormone, catecholamines gonadotrophines, PMSG, trophic hormones, prolactin, oxytocin, dopamine, bovine somatotropin, leptins and the like), growth factors (e.g. EGF, PDGF, NGF, IGF, and the like), cytokines (e.g. interleukines, CSF, G-CSF, GMCSF, EPO, TNF, TGFa, TGFb, interferons and the like), enzymes (e.g. tissue plasminogen activator, streptokinase, cholesterol biosynthetic or degradative, steroidogenic enzymes, kinases, phosphodiesterases, methylases, de-methylases, dehydrogenases, cellulases, proteases, lipases, phospholipases, aromatases, cytochromes, adenylate or guanylate cyclases, neuraminidases and the like), hormones or other receptors (e.g. steroid protein, peptide, lipid or prostaglandin, and the like), binding proteins (e.g. steroid binding proteins, growth hormone or growth factor binding proteins, and the like), immune system proteins (e.g.

antibodies, antibody fragments, chimeric antibodies, variable regions, or the like, or MHC genes), antigens (e.g. bacterial, parasitic, viral, allergens, antigens in autoimmune diseases, transplantation antigens, and the like), translation or transcription factors, oncoproteins or proto-oncoproteins, milk proteins (e.g. caseins, lactalbumin, whey, and the like), muscle proteins (e.g. myosin, tropomyosin, and the like), myeloproteins, neuroactive peptides (e.g. enkephalins), collagen, anti-sepsis peptides (e.g. BPI (bactericidal permeability-increasing protein) or tumor growth suppressing proteins or peptides, for example angiostatin or endostatin, both of with inhibit angiogenesis.

Expression of Bactericidal Permeability-Increasing (BPI) Protein in Plants
The present invention also relates to the expression of BPI, or fragments thereof, in transgenic plants, in particular in subcellular organelles, preferably in vacuoles or more preferably in plant plastids. BPI is a protein isolated from the granules of mammalian polymorphonuclear leukocytes (PMN) which are blood cells that are essential in the defense against invading microorganisms in mammals (US patent 5,641,874; Wilde et al. (1994) J. Biol. Chem. 269: 17,411-17,416). BPI is a potent bactericidal agent active against a broad range of gram-negative bacterial species (see for example US patents 5,753,620, 5,827,816 and 5,763,567, incorporated herein by reference in their entirety). It exhibits a high degree of specificity in its cytotoxic effect, i.e. 10-40 nM (0.5-2.0 micrograms), producing greater than 90% killing of 107 sensitive bacteria whereas 100-fold higher concentrations of BPI are non-toxic for other microorganisms and eukaryotic cells. BPI isolated from both human and rabbit PMN has been purified to homogeneity. The molecular weight of human BPI is approximately 58,000 Daltons (58 kDa) and that of rabbit BPI is approximately 50 kDa. Due to its exquisite selectivity and lack of cytotoxicity toward cells other than gram-negative bacteria, the BPI fragments of the present invention are particularly useful as specific therapeutic agents. Currently gram-negative bacterial infections, such as those caused by *Escherichia coli*, various species of Salmonella, Klebsiella or Pseudomonas are treated with antibiotics, such as penicillin derivatives, aminoglycosides and chloramphenicol. The effectiveness of antibiotics is limited due to the fact that gram-negative bacilli tend to display significant intrinsic resistance to many currently available antibiotics and to readily develop further resistance due to the acquisition of resistance factor plasmids. However, production of BPI in recombinant systems has been problematic and purification from mammalian cells is not cost-effective given that doses in

the gram range are preferable for therapeutic efficacy. One reason for the high dose required is the rapid clearance rate of BPI from the bloodstream. It is therefore a preferred embodiment of the present invention to express a DNA molecule encoding BPI or a BPI fragment from the plastid genome of a plant. Expression in plastids with its high yields is particularly advantageous in this case.

When employed to treat bacteremia (i.e. the presence of bacteria in the blood stream) or sepsis (bacterial contamination of bodily fluids) caused by gram-negative bacteria, BPI or BPI fragments produced according to the present invention are preferably administered parenterally, and most preferably intravenously in amounts broadly ranging between about 1 microgram and 1000 micrograms and preferably between 10 and about 250 micrograms per treatment. The duration and number of treatments may vary from individual to individual, depending upon the severity of the illness. A typical treatment regime may comprise intravenous administration of about 100 micrograms of the BPI fragments three times a day. To help avoid rapid inactivation, BPI or BPI fragments may be coupled with physiologically-acceptable carriers, such as normally occurring serum proteins (e.g. albumin or lysozyme). The BPI or BPI fragments of the present invention can also be employed topically to treat mammals suffering from skin infections caused by susceptible gram-negative bacteria which occur in bedridden patients suffering from decubitus ulcers (bed sores) or in burn patients. In a preferred embodiment, the BPI or BPI fragments of the present invention in amounts ranging between 1 microgram and 1000 micrograms per dose, may be mixed with antibiotics. In another preferred embodiment of the present invention, pharmaceutical formulations for treating mammals suffering from gram-negative bacterial infections may contain the BPI fragments of the present invention in addition to standard amounts (well-known in the art) of antibiotics such as Penicillin-G (available from E. R. Squibb and Sons, Inc., Princeton, N.J.) or cephalosporins (available from Eli Lily & Co., Indianapolis, Ind.). In a particularly preferred embodiment, the BPI fragments of the present invention may be mixed with hydrophobic antibiotics, such as rifampicin, and hydrophobic penicillins such as Penicillin-V Benzathine (Lederle Labs, Pearl River, N.Y.). The increased permeability of gram-negative bacteria after BPI treatment is expected to enhance the effectiveness of such antibiotics which cannot easily enter non-permeabilized bacteria.

The BPI or BPI fragments of the present invention are expected to be ideally-suited for co-treatment using any antibiotic, immune system cells or factors such as T-cells or interleukin-2, cytotoxic agents or the like, effective against gram-negative bacteria. Because of the increased sensitivity to the fragments of the present invention of the more pathogenic,

smooth, gram-negative bacteria, the BPI fragments of the present invention are expected to decrease resistance of such bacteria to such factors. Substantially simultaneous administration of the fragments of the present invention and the antibiotic of choice is preferred. In another preferred embodiment, BPI or BPI fragments are ingested orally according to the embodiments described *supra*, preferably to treat bacterial infections of the digestive system. The protein is administered as a pharmaceutical composition or is ingested as a medical food.
BPI has also been shown to be active in the treatment of conditions including the neutralization of the anti-coagulant activity of heparin, inhibition of angiogenesis, tumor and endothelial cell proliferation, and treatment of chronic inflammatory diseases (see US patent US5807818, incorporated herein by reference).

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by J. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory manual, Cold Spring Harbor laboratory, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. *et al.*, Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

Example 1: Isolation of the *Amb a I.1* cDNA from short ragweed (*Ambrosia artemisiifolia*) pollen by RT-PCR amplification

Total RNA from 100 mg of defatted pollen (Greer Laboratories) is isolated by the phenol/SDS method (Current Protocols in Molecular Biology). First-strand cDNA from 0.5 mg of total RNA is synthesized with the Advantage RT-for-PCR kit (Clontech) using an oligo $(dT)_{18}$ primer. The single-stranded DNA is used as a template for PCR amplification of the *Amb a I.1* cDNA (Rafnar et al. (1991) J. Biol. Chem. 266: 1229-1236) with the *Pfu Turbo*

DNA Polymerase kit (Stratagene) using the following oligonucleotides: the "top strand" primer adds a *NcoI* site to the translational start site of *Amb a I.1* (5'-GCG GCC ATG GGG ATC AAA CAC TGT TGT TA-3', SEQ ID NO:1) and the "bottom strand" primer adds a *XbaI* site after the stop codon in the 3' untranslated region (5'-GCG GTC TAG ATC ATT ATA AGT GCT TAG T-3', SEQ ID NO:2). The 1.2 kb band is digested with *NcoI* and *XbaI* for subcloning and the entire cDNA is sequenced and compared to GenBank accession number M80558. Seven bp differences are observed but none of the differences changes the amino acid composition.

Example 2: Construction of vector for homologous recombination into the tobacco plastid genome

The *trnV* and *rps12/7* intergenic region of the tobacco plastid genome is modified for insertion of chimeric genes by homologous recombination. A 1.78 kb region (positions 139255 to 141036, Shinozaki et al., (1986) *EMBO J* 5: 2043–2049) is PCR amplified from the tobacco plastid genome and a *PstI* site is inserted after position 140169, yielding 915 bp and 867 bp of flanking plastid DNA 5' and 3' of the *PstI* insertion site. PCR amplification (*PfuTurbo* DNA Polymerase, Stratagene, La Jolla, CA) is performed with a primer pair inserting a *BsiEI* site before position 139255 (5'-TAA CGG CCG CGC CCA ATC ATT CCG GAT A-3', SEQ ID NO:3) and a *PstI* site after position 140169 (5'-TAA CTG CAG AAA GAA GGC CCG GCT CCA A-3', SEQ ID NO:4). PCR amplification is also performed with a primer pair inserting a *PstI* site before position 140170 (5'-CGC CTG CAG TCG CAC TAT TAC GGA TAT G-3', SEQ ID NO:5) and a *BsiWI* site after position 141036 (5'-CGC CGT ACG AAA TCC TTC CCG ATA CCT C-3', SEQ ID NO:6). The *PstI* – *BsiEI* fragment is inserted into the *PstI* – *SacII* sites of pBluescript SK+ (Stratagene), yielding pAT216 and the *PstI-BsiWI* fragment is inserted into the *PstI-Acc65I* sites of pBluescript SK+, yielding pAT215. PAT218 contains the 1.78 kb of plastid DNA with a *PstI* site for insertion of chimeric genes and selectable markers and is constructed by ligation of the 2.0 kb *PstI-ScaI* fragment of pAT215 and the 2.7 kb *PstI-ScaI* band of pAT216.

Example 3: Amplification of the tobacco 16S rRNA gene promoter and rbs of the *rbcL* gene

The 16S rRNA gene promoter is PCR amplified from tobacco DNA (*N. tabacum* cv. Xanthi) and fused to a synthetic ribosome binding site (rbs) of the tobacco plastid *rbcL* gene. The

"top strand" primer inserts an *EcoRI* site at the 5' end of the 16S rRNA gene promoter before position 102568 (5'-GCC AGA ATT CGC CGT CGT TCA ATG AGA ATG-3', SEQ ID NO:7). The "bottom strand" primer amplifies up to position 102675 of the 16S rRNA gene promoter, removes two upstream ATG's by changing positions 102661 (A to C) and 102670 (A to C), adds the rbs of the *rbc*L gene (positions 57569-57584) as a 5' extension of the primer and inserts a *BspHI* site at the 3' end of the rbs (5'-GCC TTC ATG ATC CCT CCC TAC AAC TAT CCA GGC GCT TCA GAT TCG-3', SEQ ID NO:8). The 142 bp amplification product is gel purified and cleavage with *Eco*RI and *Bsp*HI yields a 128 bp fragment containing the tobacco 16S rRNA gene promoter fused to the rbs of the *rbc*L gene.

Example 4: Isolation of the *Arabidopsis* 16S rRNA gene promoter region

Isolation of the Arabidopsis 16S rRNA gene promoter region is facilitated by the likelihood that gene order in the *Arabidopsis* plastid genome is conserved relative to that of *Nicotiana tabacum*, a plant for which the entire plastid genome is known. In *Sinapis alba*, a closely related species to *Arabidopsis*, the 16S rRNA gene and valine tRNA are oriented as in tobacco (GenBank accession number CHSARRN1). The *Arabidopsis* 16S rRNA gene promoter region is isolated by PCR amplification (*PfuTurbo* DNA Polymerase, Stratagene, La Jolla, CA) using total *A. thaliana* (cv "Landsberg erecta") as template and the following primers that are conserved in both *Nicotiana* and *Sinapis alba*: "top strand" primer (5'-CAG TTC GAG CCT GAT TAT CC-3', SEQ ID NO:9) and the "bottom strand" primer (5'-GTT CTT ACG CGT TAC TCA CC-3', SEQ ID NO:10). The predicted 379 bp amplification product comprising the *Arabidopsis* 16S rRNA gene promoter region corresponding to nucleotides 102508 to 102872 of the tobacco plastid genome (Shinozaki et al. (1986) *EMBO J* 5: 2043–2049) is blunt end ligated into the *Eco*RV site of pGEM5Zf(-) (Promega) and sequence analysis and comparisons to the tobacco 16S rRNA gene promoter is performed.

Example 5: Amplification of the tobacco plastid *rps*16 gene 3' untranslated RNA sequence (3' UTR)

The tobacco plastid *rps*16 3' UTR is PCR amplified from tobacco DNA (*N. tabacum* cv. Xanthi) using the following oligonucleotide pair: a *Spe*I site is added immediately after the stop codon of the plastid *rps*16 gene encoding ribosomal protein S16 with the "top strand" primer (5'-CGC GAC TAG TTC AAC CGA AAT TCA AT-3', SEQ ID NO:11) and a *Pst*I site is added at the 3' end of the *rps*16 3' UTR with the "bottom strand" primer (5'-CGC TCT GCA

GTT CAA TGG AAG CAA TG-3', SEQ ID NO:12). The amplification product is gel purified and digested with *SpeI* and *PstI*, yielding a 163 bp fragment containing the tobacco *rps16* 3' UTR (positions 4941 to 5093 of the tobacco plastid genome, Shinozaki et al. (1986) *EMBO J* 5: 2043–2049) flanked 5' by a *SpeI* site and 3' with a *PstI* site.

Example 6: Construction of a 16S rRNA gene promoter :: *aadA* gene :: *rps*16 3' UTR cassette for plastid transformation selection

The coding sequence of the *aadA* gene, a bacterial gene encoding the enzyme aminoglycoside 3" adenyltransferase that confers resistance to spectinomycin and streptomycin, is isolated from pRL277 (Black et al. (1993) Molecular Microbiology 9:77-84 and Prentki et al. (1991) Gene 103: 17-23). The 5' major portion of the *aadA* coding sequence is isolated as a 724 bp *Bsp*HI-*Bss*HII fragment from pRL277 (the starting codon is at the *Bsp*HI site) and the 3' remainder of the *aadA* gene is modified by adding a *Spe*I site 20 bp after the stop codon by PCR amplification using pRL277 as template and the following oligonucleotide pair: the "top strand" primer (5'-ACC GTA AGG CTT GAT GAA-3', SEQ ID NO:13) and the "bottom strand" primer which added a *Spe*I site (5'-CCC ACT AGT TTG AAC GAA TTG TTA GAC-3', SEQ ID NO:14). The 658 bp amplification product is gel purified, digested with *Bss*HII, *Spe*I and the 89 bp fragment is ligated to the 5' portion of the *aadA* gene carried on a 724 bp *Bsp*HI-*Bss*HII fragment, the 16S rRNA gene promoter and rbs of *rbc*L carried on a 128 bp *Eco*RI-*Bsp*HI PCR amplified fragment and *Eco*RI-*Spe*I digested pLITMUS28 vector (New England Biolabs), yielding pAT223. A three-way ligation is performed on an *Eco*RI- *Spe*I 0.94 kb fragment of pAT223 containing the 16S rRNA gene promoter-rbs driven *aadA* gene, a 163 bp *Spe*I, *Pst*I digested PCR fragment containing the *rps*16 3' UTR and pUC19 (New England Biolabs) cut with *Eco*RI, *Pst*I to obtain pAT229 containing the 16S rRNA gene promoter driving the *aadA* gene with the *rps*16 3' UTR.

Example 7: Amplification of the bacteriophage T7 gene 10 promoter

The bacteriophage T7 gene 10 promoter is PCR amplified from pET-3d (Stratagene) using the following oligonucleotide pair: the "top strand" primer inserts an *Eco*RI site at the 5' end of the T7 promoter (5'-CCC GAA TTC ATC CCG CGA AAT TAA TA-3', SEQ ID NO:15) and the "bottom strand" primer inserts a *Nco*I site at the 3' end (5'-CGG CCA TGG GTA TAT CTC CTT CTT AAA GTT AAA-3', SEQ ID NO:16). The amplification product is gel purified and cleavage with *Eco*RI, *Nco*I produces a 96 bp fragment containing the T7 promoter.

Example 8: Amplification of the bacteriophage T7 gene 10 terminator

The bacteriophage T7 gene 10 terminator is PCR amplified from pET-3d (Stratagene) using the following oligonucleotide pair: the "top strand" primer inserts a *HindIII* site at the 5' end of the terminator (5'-GCG AAG CTT GCT GAG CAA TAA CTA GCA TAA-3', SEQ ID NO:17) and the "bottom strand" primer inserts a *PstI* site at the 3' end of the terminator (5'-GCG CTG CAG TCC GGA TAT AGT TCC TCC T-3', SEQ ID NO:18). The amplification product is gel purified and cleavage with *HindIII-PstI* produces an 86 bp fragment containing the T7 terminator.

Example 9: Amplification of the *Arabidopsis thaliana* plastid *psbA* 3' untranslated RNA sequence (UTR)

The *A. thaliana* plastid *psbA* 3' UTR is PCR amplified from *A. thaliana* DNA (ecotype Landsberg erecta) using the following oligonucleotide pair: the "top strand" primer adds a *Spe*I site to the 5' end of the 3' UTR and eliminates a *Xba*I site in the native sequence by mutating a G to an A (underlined) (5'-GCG ACT AGT TAG TGT TAG TCT AAA TCT AGT T-3', SEQ ID NO:19) and the "bottom strand" primer adds a *Hind*III site to the 3' end of the UTR (5'-CCG CAA GCT TCT AAT AAA AAA TAT ATA GTA-3', SEQ ID NO:20). The amplified region extends from position 1350 to 1552 of GenBank accession number X79898. The 218 bp PCR product is gel purified, digested with *Spe*I and *Hind*III and ligated with the *Hind*III-*Pst*I cut PCR fragment carrying the T7 terminator into the *Spe*I-*Pst*I sites of pBluescript SK- (Stratagene), yielding pPH171. Sequence analysis of the *psbA* 3' UTR region of pPH171 compared to GenBank accession number X79898 reveals deletion of adenine nucleotides at positions 1440 and 1452.

Example 10: Construction of a vector using a polyguanosine tract as a substitute for a 3' UTR

A polyguanosine tract has been shown to substitute functionally for the plastid *atpB* gene 3' UTR *in vivo* (Drager et al. (1996) RNA 2:652-663). A poly G tract containing 18 consecutive guanosine residues flanked by *Spe*I, *Hind*III sticky ends on the 5' and 3' ends respectively is assembled by annealing the following two kinased oligonucleotides: (5'-CTA GTG GGG GGG GGG GGG GGG GGA-3', SEQ ID NO:21) and (5'-AGC TTC CCC CCC CCC CCC CCC CCA-3', SEQ ID NO:22). The polyG$_{18}$ tract containing *Spe*I, *Hind*III sticky ends is

ligated with the *Hind*III, *Pst*I digested PCR fragment containing the T7 terminator into the *Spe*I, *Pst*I sites of pBluescript SK+ (Stratagene), yielding pAT222.

Example 11: Preparation of a chimeric gene containing the ragweed pollen allergen *Amb a I.1* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

A bacteriophage T7 gene 10 promoter :: *Amb a I.1* :: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed by a four-way ligation of the 96 bp *Eco*RI, *Nco*I PCR fragment containing the T7 promoter, the 1.2 kb *Nco*I, *Xba*I PCR fragment containing the *Amb a I.1* cDNA and the 295 bp *Xba*I, *Pst*I fragment of pPH171 containing the A. thaliana *psbA* 3' UTR and T7 terminator into the *Eco*RI, *Pst*I sites of pGEM-3Z (Stratagene), yielding plasmid pAT230. The T7 promoter driven *Amb a I.1* gene cassette is ligated to the *aadA* selectable marker cassette by cloning the 1.1 kb *Hind*III, *Eco*RI fragment of pAT229 containing the 16S rRNA gene promoter-rbs :: *aadA* :: *rps*16 3' UTR cassette and the 1.6 kb *Eco*RI, *Pst*I pAT230 fragment carrying the T7 promoter :: *Amb a I.1* :: *psbA* 3' UTR :: T7 terminator cassette into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene), producing plasmid pAT234. Plastid transformation vector pAT238 is constructed by ligating the 2.7 kb *Pst*I band from pAT234 containing the *Amb a I.1* and selectable marker cassettes into the *Pst*I site of pAT218 and screening for an insert orientation where the *Amb a I.1* gene is transcribed in the same direction as the *rps12/7* ORF.

Example 12: Preparation of a chimeric gene containing the ragweed pollen allergen *Amb a I.1* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and a polyguanosine tract in a tobacco plastid transformation vector

The plastid transformation vector pAT239 containing the T7 gene 10 promoter :: *Amb a I.1* :: polyguanosine tract :: T7 terminator and the 16S rRNA gene promoter-rbs :: *aadA* :: *rps*16 3' UTR cassette is constructed by a four-way ligation of the 96 bp *Eco*RI, *Nco*I PCR fragment containing the T7 promoter, the 1.2 kb *Nco*I, *Xba*I PCR fragment containing the *Amb a I.1* cDNA and the 30 bp *Xba*I, *Hind*III fragment of pAT222 carrying the poly G_{18} tract into the 5.9 kb *Eco*RI, *Hind*III vector fragment of pAT238 containing the selectable marker, T7 terminator and flanking homologous plastid regions and screening for an insert

orientation where the *Amb a I.1* gene is transcribed in the same direction as the *rps12/7* ORF.

Example 13: Preparation of a chimeric gene containing the *Dermatophagoides* allergen *Der f I* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the *Derf I* gene is described in US patent 5,770,202. This sequence is used to design PCR primers for amplification of the *Der f I* coding sequence from *Dermatophagoides farinae* cDNA. The cDNA is obtained by reverse transcription (as for *Amb a I.1*) from total RNA extracted from a *D. farinae* dust mite preparation (Greer Laboratories). A bacteriophage T7 gene 10 promoter :: *Der f I* :: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed as described for pAT230. The T7 promoter driven *Der f I* gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 14: Preparation of a chimeric gene containing the *Dermatophagoides* allergen *Der f II* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the *Der f II* gene is described in US patent 5,770,202. This sequence is used to design PCR primers for amplification of the *Der f II* coding sequence from *Dermatophagoides farinae* cDNA. The cDNA is obtained by reverse transcription (as for *Amb a I.1*) from total RNA extracted from a *D. farinae* dust mite preparation (Greer Laboratories). A bacteriophage T7 gene 10 promoter :: *Der f II* :: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed as described for pAT230. The T7 promoter driven *Der f II* gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 15: Preparation of a chimeric gene containing the *Dermatophagoides* allergen *Der p I* coding sequence fused to a bacteriophage T7 gene 10 promoter and

terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the *Der p I* gene is described in US patent 5,770,202. This sequence is used to design PCR primers for amplification of the *Der p I* coding sequence from *Dermatophagoides pteronyssinus* cDNA. The cDNA is obtained by reverse transcription (as for *Amb a I.1*) from total RNA extracted from a *D. pteronyssinus* dust mite preparation (Greer Laboratories). A bacteriophage T7 gene 10 promoter :: *Der p I* :: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed as described for pAT230. The T7 promoter driven *Der p I* gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 16: Preparation of a chimeric gene containing the *Dermatophagoides* allergen *Der p II* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the *Der p II* gene is described in US patent 5,770,202. This sequence is used to design PCR primers for amplification of the *Der p II* coding sequence from *Dermatophagoides pteronyssinus* cDNA. The cDNA is obtained by reverse transcription (as for *Amb a I.1*) from total RNA extracted from a *D. pteronyssinus* dust mite preparation (Greer Laboratories). A bacteriophage T7 gene 10 promoter :: *Der p II* :: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed as described for pAT230. The T7 promoter driven *Der p II* gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 17: Preparation of a chimeric gene containing the Johnson grass pollen allergen *Sor h I* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the *Sor h I* gene is described in US patent 5,480,972. A bacteriophage T7 gene 10 promoter :: *Sor h I* :: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed. The T7 promoter driven *Sor h I* gene cassette is ligated to the

aadA selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 18: Preparation of a chimeric gene containing the birch pollen allergen *Bet V I* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the *Bet V I* gene is described in Breiteneder et al. (1989) EMBO J. 8: 1935-1938. A bacteriophage T7 gene 10 promoter :: *Bet V I* :: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed. The T7 promoter driven *Bet V I* gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 19: Preparation of a chimeric gene containing the mosquito salivary allergen *rAed a 1* coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the *rAed a 1* gene is described in WO 98/04274. A bacteriophage T7 gene 10 promoter :: *rAed a 1*:: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed. The T7 promoter driven *rAed a 1* gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 20: Preparation of a chimeric gene containing the glutamic acid decarboxylase (GAD) coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the human GAD gene is obtained from Genbank (accession number L16888). A bacteriophage T7 gene 10 promoter :: GAD:: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed. The T7 promoter driven GAD gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of

pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 21: Preparation of a chimeric gene containing the thyroglobulin coding sequence fused to a bacteriophage T7 gene 10 promoter and terminator and the *Arabidopsis* plastid *psbA* 3' UTR in a tobacco plastid transformation vector

The coding sequence of the thyroglobulin gene is obtained from Genbank (accession number U93033). A bacteriophage T7 gene 10 promoter :: thyroglobulin:: *A. thaliana psbA* 3' UTR :: T7 terminator cassette is constructed. The T7 promoter driven thyroglobulin gene cassette is ligated to the *aadA* selectable marker cassette and inserted into the *Hind*III, *Pst*I sites of pBluescript SK+ (Stratagene). A plastid transformation vector is then constructed as described in example 11.

Example 22: Biolistic Transformation of the Tobacco Plastid Genome

Seeds of *Nicotiana tabacum* c.v. 'Xanthi nc' are germinated seven per plate in a 1" circular array on T agar medium and bombarded 12-14 days after sowing with 1 μm tungsten particles (M10, Biorad, Hercules, CA) coated with plasmid DNA essentially as described in Svab, Z. and Maliga, P. (1993) *PNAS* 90, 913–917. Bombarded seedlings are incubated on T medium for two days after which leaves are excised and placed abaxial side up in bright light (350-500 µmol photons/m^2/s) on plates of RMOP medium (Svab, Z., Hajdukiewicz, P. and Maliga, P. (1990) *PNAS* 87, 8526–8530) containing 500 µg/ml spectinomycin dihydrochloride (Sigma, St. Louis, MO). Resistant shoots appearing underneath the bleached leaves three to eight weeks after bombardment are subcloned onto the same selective medium, allowed to form callus, and secondary shoots isolated and subcloned. Complete segregation of transformed plastid genome copies (homoplasmicity) in independent subclones is assessed by standard techniques of Southern blotting (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor). BamHI/EcoRI-digested total cellular DNA (Mettler, I. J. (1987) *Plant Mol Biol Reporter* 5, 346–349) is separated on 1% Tris-borate (TBE) agarose gels, transferred to nylon membranes (Amersham) and probed with ^{32}P-labeled random primed DNA sequences corresponding to a 0.7 kb BamHI/HindIII DNA fragment from pC8 containing a portion of the *rps7/12* plastid targeting sequence (see patent application WO 98/11235).

Homoplasmic shoots are rooted aseptically on spectinomycin-containing MS/IBA medium (McBride, K. E. et al. (1994) *PNAS* 91, 7301-7305) and transferred to the greenhouse.

Example 23: Construction of a plastid-targeted bacteriophage T7 RNA polymerase gene fused to the tobacco PR-1a promoter

A synthetic oligonucleotide linker comprising an *NcoI* restriction site and ATG start codon followed by the first seven plastid transit peptide codons from the *rbcS* gene (encoding the small subunit of ribulose bisphosphate carboxylase) and endogenous *PstI* restriction site (top strand: 5'-CAT GGC TTC CTC AGT TCT TTC CTC TGC A-3', SEQ ID NO:23; bottom strand: 5'-GAG GAA AGA ACT GAG GAA GC-3', SEQ ID NO:24), a 2.8 kb *PstI/SacI* DNA fragment of pCGN4205 (McBride, K. E. et al. (1994) PNAS 91, 7301-7305) containing the bacteriophage T7 RNA polymerase gene (T7 Pol) fused in frame to the 3' portion of the *rbcS* gene transit peptide coding sequence, a 0.9 kb *NcoI/KpnI* DNA fragment of pCIB296 containing the tobacco PR-1a promoter with an introduced *NcoI* restriction site at the start codon (Uknes et al. (1993) Plant Cell 5, 159-169) and 4.9 kb *SfiI/KpnI* and 6.6 kb *SacI/SfiI* fragments of binary Agrobacterium transformation vector pSGCGC1 (a derivative of pGPTV-Hyg containing the polylinker from pGEM4 (Promega, Madison WI) cloned into the *SacI/HindIII* sites) are ligated to construct pPH110.

Example 24: Introduction of the chimeric PR-1a / T7 Pol gene into the tobacco nuclear genome by *Agrobacterium*-mediated leaf disc transformation

Hygromycin resistant NT-pPH110 tobacco plants are regenerated as described from shoots obtained following cocultivation of leaf disks of N. tabacum 'Xanthi' and "NahG" (Friedrich *et al.* (1995) *Plant Mol Biol* 29:959-68) with GV3101 *Agrobacterium* carrying the pPH110 binary vector. For each transgenic line duplicate leaf punches of approximately 2-3 cm^2 are incubated for 2 days in 3 ml of BTH (5.6 mg/10 ml) or sterile distilled water under ca. 300 $\mu mol/m^2/s$ irradiance. Leaf material is harvested, flash frozen and ground in liquid nitrogen. Total RNA is extracted (Verwoerd et al. (1989) NAR 17, 2362) and Northern blot analysis is carried out as described (Ward et al. (1991) The Plant Cell 3, 1085-1094) using a radiolabelled T7 RNA polymerase gene probe. Plants of nineteen NT-110X (Xanthi genetic background) and seven NT-110N (NahG genetic background) T1 lines showing a range of T7 Pol expression are transferred to the greenhouse and self pollinated. Progeny

segregating 3:1 for the linked hygromycin resistance marker are selfed and homozygous T2 lines selected.

Example 25: Induction of ragweed allergen expression in plastids of transgenic plants

Homozygous NT-110X and NT-110N plants containing the PR-1a-T7 RNA Pol construct are used to pollinate homoplasmic plastid transformant lines carrying the maternally inherited pAT238 and pAT239 constructs. The Nt_ pAT238 x NT-110X or NT_110N, and Nt_pAT239 x NT-110X or NT_110N F1 progeny (which were heterozygous for the PR-1/T7 polymerase nuclear expression cassette and homoplasmic for the T7/ *Amb a I.1*_plastid expression cassette) are germinated on soil. Upon reaching a height of 20-40 cm, the plants are sprayed with the inducer compound BTH to elicit T7 Pol-regulated expression of the *Amb a I.1* gene that is localized to the plastids. Plant material is harvested just prior to induction and at 8 hours and 1, 2, 3, 7, and 14 or 28 days following induction and flash frozen in liquid nitrogen. Similar procedures are applied for transgenic plants comprising the *Der f I, Sor h I, Bet V I* and *rAed a 1* allergen genes, and the GAD and thyroglobulin genes.

Example 26: Determination of antigen expression and content of transgenic plants

Total RNA is extracted (Verwoerd et al. (1989) NAR 17, 2362) and Northern blot analysis is carried out as described (Ward et al. (1991) The Plant Cell 3, 1085-1094) using radiolabelled probes specific for the *Amb a I.1* gene. In order to determine the amount of *Amb a I.1* present in the tissues of transgenic plants, chemiluminescent (Amersham) Western blot analysis is performed according to the manufacturer's instructions and Harlow and Lane (1988) Antibodies: A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor using antisera raised against the *Amb a I.1* proteins and purified *Amb a I.1* protein standards. Similar procedures are applied for the *Der f I, Sor h I* and *Bet V I, rAed a 1* allergens, and for GAD and thyroglobulin.

Example 27: Induction of oral tolerance in rats

Female Lewis or Wistar Furth rats weighing 150 to 220 g (6-8 weeks of age) are used. Rats are immunized in both hind footpads with 50 µg guinea pig antigen emulsified in complete Freund's adjuvant (CFA). In some experiments, 50 µg ovalbumin (OVA) (Sigma) is added to the emulsified antigen and injected similarly. For induction of oral tolerance, rats are fed transgenic plants of the present invention five times at three-day intervals. Nine days after

immunization, the rats are sacrificed and their popliteal lymph nodes are removed. A single cell suspension is prepared by pressing the lymph nodes through a stainless steel mesh. A total of 105 lymph node cells (LNC) are cultured with the indicated number of either irradiated (2000 Rads) or intact LNC derived from fed rats in quadruplicate in round-bottomed 96-well plate (Costar). Antigens (50 μg/ml) are added to the culture in a volume of 20 μl. The cultures are incubated for 80 hours and are pulsed with 1 μCi [3 H] TdR/well for the last 16 hours of culture. The cultures are then harvested on an automatic cell harvester and read on a standard liquid scintillation counter. The percentage of primed LNC (PLNC) proliferation is then calculated. Proliferation Media RPMI (Gibco) is used in all the experiments. The medium is filtered sterile after adding 2x10-5 M 2-mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% non-essential amino acids, and 1% autologous serum.

Example 28: Measurement of antibodies

A. Serum Levels of Antibodies

A solid-phase enzyme-linked immunoabsorbent assay (ELISA) is used for determination of antibody titers against the antigen. Microtiter plates are incubated with 0.1 ml per well of 10 μg antigen/ml in doubled distilled water. Plates are incubated for 18 hrs at 25° C. After 3 washes with PBS/Tween-20 (Bio-Rad), pH 7.5, plates are incubated with 3% BSA/PBS for 2 hrs at 37° C, washed twice, and 100 μl of diluted serum is added in quadruplicate. The plates are incubated for 2 hrs at 37° C. After three rinses with PBS/Tween-20, plates are incubated with 100 μl/well of peroxidase-conjugated goat anti-rat IgG antibody (Tago, USA) diluted 1:1000 in 1% BSA/PBS for 1 hr at 25° C. Color reaction is obtained by exposure to D-phenylenediamine (0.4 mg/ml phosphate) citrate buffer, pH 5.0) containing 30% H2O2. The reaction is stopped by adding 0.4N H2 SO_4 and OD 492 nm is read on an ELISA reader.

B. In Vitro Measurement of Antibody Production:

Popliteal and splenic LNC are obtained from fed, naive and challenged rats and seeded at a concentration of 107 cells per ml petri dish either alone or irradiated (2000 Rads) together with other PLNC as indicated. The cultures are maintained in proliferation media, with or without antigen (20 μg/ml), for 3 days in an incubator and then harvested. The diluted

supernatants are used to examine the in vitro production and secretion of IgG antibody and are measured for antibody production using an ELISA test as described previously.

Example 29: Induction of oral tolerance in mice

Female Balb/c mice aged 6 to 8 weeks are used. For induction of oral tolerance, mice are fed extracts from tobacco plants expressing recombinant ragweed from the T7/ *Amb a I.1* plastid expression cassette according to Example 22. More specifically, mice are fed Fraction 1 or Fraction 2 of tobacco plants transformed with the T7/ *Amb a I.1* plastid expression cassette or, as a control, Fraction 1 or Fraction 2 from untransformed, control tobacco plants. Naïve animals are divided into three recombinant ragweed-fed groups (0.1, 1, and 10 mg recombinant ragweed/day) and one untransformed tobacco-fed group (as control). Each group consists of 8 animals. Oral administration of antigens to the animals starts after an initial 10 day period of acclimatization to the vivarium facility. Animals are given by gavage the corresponding tobacco fraction adjusted to a final volume of 0.2-0.5 ml with H_2O or phosphate-buffered saline once daily for 5-8 sequential days.

Induction of oral tolerization is assessed four days after the last oral feeding of tobacco-expressed allergen. All four groups of mice are sensitized three times by intraperitoneal injection of recombinant ragweed (10 μg/animal) at two-week intervals. In addition to recombinant ragweed, each mouse is also sensitized to ovalbumin (10 μg/animal; Sigma Chemical, St. Louis, MO, USA) to demonstrate that the mice are capable of mounting an immune response to other antigens. Antigens are dissolved in 0.9% saline and mixed with aluminum hydroxide (2:1; Alu Gel S Serva, Feinbiochemica GmbH, Heidelberg, Germany) to give the desired concentration in a final volume of 200 μl/mouse.

Serum samples for determination of antigen-specific IgE, IgG1, and IgG2a levels are collected four times: once prior to start of oral administration of antigen and at appropriate time points for each of the three immunizations. Sera are aliquoted and stored at –20 ^{o}C until assayed.

All serum samples are subjected to capture, solid-phase enzyme-linked immunosorbent assay (ELISA) for determination of recombinant ragweed and ovalbumin-specific serum IgE, IgG1, and IgG2a levels. Ninety-six well microtiter plates (Immunoplates Maxisorp™ Surface, Nunc, Roskilde, Denmark) are incubated with 0.1 ml per well of corresponding antigen (1-10 μg/ml) in 0.015 M sodium carbonate bicarbonate buffer (pH 9) for 2 h at 37 ^{o}C followed by overnight at 4 ^{o}C. All washings are performed with 0.8 % salt solution

containing 0.05% Tween-solution (pH 7.4; washing buffer; Sigma Chemical). Unless noted, serum samples, revealing antibodies, and reagents are diluted in washing buffer containing 1 % heat-inactivated fetal calf serum, all incubations are performed in a humid atmosphere, and the reaction volume is 100 µl/well. After coating, the plates are washed three times in washing buffer. Serum samples are applied in three-fold serial dilutions to the antigen-coated wells and incubated at 37 ^{o}C for 2 h. After washing three times in washing buffer, corresponding biotinylated revealing antibodies (So. Biotechnology Assoc., Birmingham, AL, USA; Binding Site, Birmingham, UK) are applied to the plates at 37 ^{o}C for 1 h. After washing, horseradish peroxidase-labeled Avidin D is applied to detect immunoreactivity (1:3,000, 60 min, 37 ^{o}C; Vector Lab., Burlingame, CA, USA). After rewashing, plates are additionally washed three times using citrate buffer (substrate buffer, pH 5). Enzyme activity is detected with *o*-phenylenediamine (Sigma Chemical) in substrate buffer (0.5 mg/ml) and hydrogen peroxide (1 µl/ml) and stopped after 1-15 min with 4 N H_2SO_4 (50 µl/well; Merck KgaA, Darmstadt, Germany). Optical densities are determined spectrophotometrically at 492 nm with an ELISA reader. Final results are expressed in relative ELISA units (REU) compared to a standard serum pool (arbitrarily assigned 100 REU) or in absolute protein concentrations (µg/ml).

Similar procedures to test for oral tolerance are applied for transgenic plants comprising the *Der f I, Sor h I, Bet v I* and *rAed a 1* allergen genes, and the GAD and thyroglobulin genes or any other antigen of the invention.

Induction of oral tolerance to house dust mite extract is described in Sato et al., Immunology 95: 193-199, 1998. House dust mite experiments in humans are described by Passalacqua et al., Lancet, 351: 629-632, 1998.

Example 30: Chemically-regulated expression of the human BPI gene in plant plastids

<u>I. Construction of a plastid transformation vector containing the BPI gene under control of a promoter element responsive to the bacteriophage T7 RNA polymerase</u>

The coding sequence of the human BPI gene (as described in US patent 5,198,541) is cloned as a 182 bp *NcoI/EcoRI* restriction fragment (containing the 5' portion of the cDNA and incorporating the start codon into the *NcoI* site) and a 1288 bp *EcoRI/XbaI* restriction fragment (containing the 3' portion of the cDNA sequence and the termination codon). These fragments are gel-purified and ligated in a three-way reaction to a 7693 bp *NcoI/XbaI* fragment from plasmid pT7_GUS. The resulting plasmid (pT7_BPI) is verified by DNA

sequencing and restriction analysis to contain the BPI gene downstream of the T7 gene 10 promoter from plasmid pET3a (Novagen) with a chimeric 5' untranslated leader containing the sequence 5'-GGG AGA CCA CAA CGG TTT CCC TCT AG-3' (SEQ ID NO:25) derived from the T7 gene 10 leader, the linker sequence 5'-CGAGG-3' derived from the *StuI* linker of plasmid pC8, and the sequence 5'-GGG AGT CCC TGA TGA TTA AAT AAA CCA AGA TTT TA<u>C</u> <u>CAT</u> <u>GG</u>-3' (SEQ ID NO:26) derived from the tobacco plastid *psbA* gene 5' leader modified to include an *NcoI* restriction site at the start codon (underlined). pT7_GUS is constructed by ligating a gel-purified 386 bp *EcoRI/NcoI* fragment of plasmid pPH142 that contains the chimeric T7 promoter/*psbA* 5' leader to a dephosphorylated and gel purified 9241 bp *NcoI/EcoRI* fragment of GUS plastid transformation vector pC8 (International Patent Application WO 98/11235). Plasmid pPH142 is constructed by digesting plasmid pPH136 with *BsaBI* (2 hr at 60° C), gel purifying and redigesting the linearized plasmid DNA with *BsmFI* (1 hr at 37° C), treating with alkaline phosphatase (1 hr at 37° C followed by phenol extraction) and ligating the resulting gel-purified 3398 bp fragment to a synthetic double-stranded oligonucleotide made by annealing the oligonucleotides T73a_U (5'-CGA TCC CGC GAA ATT AAT ACG ACT CAC TAT AGG GAG ACC ACA ACG GTT TCC C-3', SEQ ID NO:27) and T73a_L (5'-TAG AGG GAA ACC GTT GTG GTC TCC CTA TAG TGA GTC GTA TTA ATT TCG CGG GAT CG-3', SEQ ID NO:28) subsequent to phosphorylation with T4 polynucleotide kinase (1 hr at 37° C). Plasmid pPH136 DNA is amplified in an *E. coli* strain lacking dam methylase activity (DM-1, Stratagene, La Jolla, California) in order to permit digestion with BsaBI. pPH136 is in turn constructed by ligating a 3428 bp gel-purified *StuI/NcoI* fragment from plasmid pPH120 to a synthetic double-stranded oligonucleotide made by annealing the oligonucleotides minpsb_U (5'-GGG AGT CCC TGA TGA TTA AAT AAA CCA AGA TTT TAC-3', SEQ ID NO:29) and minpsb_L (5'-CAT GGT AAA ATC TTG GTT TAT TTA ATC ATC AGG GAC TCC C-3', SEQ ID NO:30). This oligonucleotide comprises a 38 nt portion of the tobacco *psbA* gene 5' untranslated leader sequence implicated in translational up-regulation of the *psbA* gene product in an in-vitro plastid translation system (Hirose and Sugiura, EMBO J. **15**: 1687-1695, 1996). pPH120 comprises a modified portion of the *psbA* promoter-driven *aadA* gene and divergent T7-*lac* promoter of plasmid pC8 (derived from Novagen plasmid pET21d and a chimeric ribosome binding site from the tobacco plastid *rbcL* gene, into which a fragment of yeast DNA is inserted in order to block the low-level bidirectional transcriptional activity of the *psbA* promoter characteristic of vectors derived from plasmid pPRV111A (Allison et al., EMBO J.

1996 Jun 3;15(11):2802-9). PPH120 is constructed by ligating a 256 bp *EcoRI/HincII* fragment of the *Saccharomyces cerevisiae* LEU2 gene, a 2645 bp *EcoRI/DraIII* fragment of pPH119, and a 569 bp *DraIII*/Klenow-filled *EcoRI* fragment of pPH119. PPH119 is in turn constructed by digesting plasmid pPH118 with *StuI* and religating in order to remove the duplicated *StuI* linker present in the pC8-derived chimeric T7 promoter of pPH118. PPH118 contains a 235 bp *SpeI/NcoI* fragment of pC8 that is isolated and gel purified and then ligated into cloning vector pGEM5Zf(+)(Promega, Madison WI) that is digested with *NcoI* and *SpeI*.

II: Biolistic transformation of the tobacco plastid genome with plasmid pT7_BPI

Transformation of the plastid genome is carried out as described in Example 22. Resistant shoots appearing underneath the bleached leaves three to eight weeks after bombardment are subcloned onto the same selective medium, allowed to form callus, and secondary shoots isolated and subcloned. Complete segregation of transformed plastid genome copies (homoplasmicity) in independent subclones is assessed by standard techniques of Southern blotting (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor). *BamHI/NcoI*-digested total cellular DNA (Mettler, I. J. (1987) *Plant Mol Biol Reporter* **5**, 346–349) is separated on 1% Tris-borate (TBE) agarose gels, transferred to nylon membranes (Amersham) and probed with ^{32}P-labeled random primed DNA sequences corresponding to a 0.7 kb *BamHI/HindIII* DNA fragment from pC8 containing a portion of the *rps7/12* plastid targeting sequence. Homoplasmic shoots containing the expected 1.25 kb fragment and lacking the wild-type 3.3 kb fragment are rooted aseptically on spectinomycin-containing MS/IBA medium (McBride, K. E. et al. (1994) *PNAS* **91**, 7301-7305) and transferred to the greenhouse.

III: Chemical induction of BPI expression in tobacco plants transformed with plasmid pT7_BPI and carrying a bacteriophage T7 RNA polymerase gene with a plastid-targeting sequence in the nuclear genome under control of the BTH-responsive PR-1a promoter.

Homoplasmic Nt_pT7_BPI plants of line C35BPI-5B-4 are pollinated by tobacco line Nt_110X6b-5 and the seed tested for maternal inheritance of the spectinomycin resistance marker carried by the transforming plasmid pT7_BPI. Additional seeds of these F1 progeny are germinated in soil in 6 inch clay pots. Eight weeks after sowing, plants are sprayed with a foliar application of 1.0 mM BTH until run-off occurs. Tissue is harvested for analysis of

BPI accumulation at intervals of 0, 1, 2, 3, 7, 14, 21, 28 and 35 days following BTH application. Control plants of the same line sprayed with water or an inert ingredient, as well as C35BPI-5B-4 plants carrying only the plastid BPI gene and not the chemically-inducible transactivator are germinated at the same time and assayed in the same manner using the same time schedule. Accumulation of BPI is assayed by standard techniques of Northern hybridization (mRNA) and Western blotting (protein). Additionally, activity assays are performed using leaf extracts in order to assess the ability of the plant-expressed BPI to inhibit the exponential growth in liquid medium of gram-negative bacteria (e.g. *E. coli*).

Example 31: Construction of Expression Cassettes for Nuclear Expression of a Transgene in Plants

Gene sequences intended for expression in transgenic plants are firstly assembled in expression cassettes behind a suitable promoter and preferably upstream of a suitable transcription terminator. All requirement for constructions of plant expression cassettes apply to DNA molecules of the present invention, in particular DNA molecules encoding a transactivator or a therapeutically active protein and are carried out using techniques well-known in the art.

Promoter Selection

The selection of promoter used in expression cassettes determines the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and this selection reflects the desired location of biosynthesis of the transgene. Alternatively, the selected promoter may drive expression of the transgener under a light-induced or other temporally regulated promoter. A further alternative is that the selected promoter be chemically regulated. This provides the possibility of inducing the expression of the transgene only when desired and caused by treatment with a chemical inducer.

3' Regulatory Sequences

A variety of 3' regulatory sequences are available for use in expression cassettes. In the plant nucleus, these sequences are for example responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators and those which are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator, and the pea

rbcS E9 terminator. These can be used in both monocotyledonous and dicotyledonous plants.

Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the transgene to increase its expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *Adh1* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective in enhancing expression of fusion constructs made with the chloramphenicol acetyltransferase gene (Callis *et al.*, Genes Develop 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression (Callis *et al.*, *supra*). Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader. A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "Ω-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (*e.g.* Gallie *et al.* Nucl. Acids Res. 15: 8693-8711 (1987); Skuzeski *et al.* Plant Molec. Biol. 15; 65-79 (1990)).

Example 32: Targeting of Gene Products within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. For example, the targeting of gene products to plastids, for example chloroplasts, is controlled by a signal sequence found at the aminoterminal end of various proteins and which is cleaved during chloroplast import yielding the mature protein (*e.g.* Comai *et al.* J. Biol. Chem. 263: 15104-15109 (1988)). These signal sequences can be fused to heterologous gene products to effect the import of heterologous products into the chloroplast (van den Broeck *et al.* Nature 313: 358-363 (1985)). DNA encoding for appropriate signal sequences can be isolated from the 5' end of the cDNAs encoding the RUBISCO protein, the CAB protein, the EPSP synthase enzyme, the GS2 protein and many

other proteins which are known to be chloroplast localized. The signal sequence selected should include the known cleavage site and the fusion constructed should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or alternatively replacement of some amino acids within the transgene sequence. Fusions constructed for chloroplast import can be tested for efficacy of chloroplast uptake by *in vitro* translation of *in vitro* transcribed constructions followed by *in vitro* chloroplast uptake using techniques described by (Bartlett *et al.* In: Edelmann *et al.* (Eds.) Methods in Chloroplast Molecular Biology, Elsevier. pp 1081-1091 (1982); Wasmann *et al.* Mol. Gen. Genet. 205: 446-453 (1986)). Accordingly, any of these signal sequences are fused to a nucleotide sequence encoding a transactivator to target the transactivator into the chloroplast. The above described mechanisms for cellular targeting are utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell targeting goal under the transcriptional regulation of a promoter which has an expression pattern different to that of the promoter from which the targeting signal derives.
Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (*e.g.* Unger *et al.* Plant Molec. Biol. 13: 411-418 (1989)). The cDNAs encoding these products can also be manipulated to effect the targeting of heterologous gene products to these organelles. Examples of such sequences are the nuclear-encoded ATPases and specific aspartate amino transferase isoforms for mitochondria. Targeting to cellular protein bodies has been described by Rogers *et al.* (Proc. Natl. Acad. Sci. USA 82: 6512-6516 (1985)). In addition sequences have been characterized which cause the targeting of gene products to other cell compartments. Aminoterminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). Additionally, aminoterminal sequences in conjunction with carboxyterminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al.* Plant Molec. Biol. 14: 357-368 (1990)).

Example 33: Examples of Expression Cassette Construction

The present invention encompasses the expression of a DNA molecule of the present invention under the regulation of any promoter which is expressible in plants, regardless of the origin of the promoter. Therefore the DNA molecule of the present invention is inserted

into any of the expression cassettes using techniques well-known in the art. These expression cassettes can then be easily transferred to the plant transformation vectors described below. Furthermore, the invention also encompasses the use of any plant-expressible promoter in conjunction with any further sequences required or selected for the expression of the transgene. Such sequences include, but are not restricted to, transcriptional terminators, extraneous sequences to enhance expression (such as introns [*e.g. Adh* intron 1], viral sequences [*e. g.* TMV-Ω]), and sequences intended for the targeting of the gene product to specific organelles and cell compartments.

Constitutive Expression: the CaMV 35S Promoter

Construction of the plasmid pCGN1761 is described in the published patent application EP 0 392 225. pCGN1761 contains the "double" 35S promoter and the *tml* transcriptional terminator with a unique *EcoRI* site between the promoter and the terminator and has a pUC-type backbone. A derivative of pCGN1761 is constructed which has a modified polylinker which includes *NotI* and *XhoI* sites in addition to the existing *EcoRI* site. This derivative is designated pCGN1761ENX. pCGN1761ENX is useful for the cloning of cDNA sequences or gene sequences (including microbial ORF sequences) within its polylinker for the purposes of their expression under the control of the 35S promoter in transgenic plants. The entire 35S promoter-gene sequence-*tml* terminator cassette of such a construction can be excised by *HindIII, SphI, SalI,* and *XbaI* sites 5' to the promoter and *XbaI, BamHI* and *BglI* sites 3' to the terminator for transfer to transformation vectors. Furthermore, the double 35S promoter fragment can be removed by 5' excision with *HindIII, SphI, SalI, XbaI,* or *PstI*, and 3' excision with any of the polylinker restriction sites (*EcoRI, NotI* or *XhoI*) for replacement with another promoter.

Expression under a Chemically Regulatable Promoter

(1) PR1-a Promoter

This section describes the replacement of the double 35S promoter in pCGN1761ENX with any promoter of choice; by way of example the chemically regulated PR-1a promoter is described. The promoter of choice is preferably excised from its source by restriction enzymes, but can alternatively be PCR-amplified using primers which carry appropriate terminal restriction sites. Should PCR-amplification be undertaken, then the promoter should be resequenced to check for amplification errors after the cloning of the amplified promoter in the target vector. The chemically regulatable tobacco PR-1a promoter is

cleaved from plasmid pCIB1004 (see EP 0 332 104) and transferred to plasmid pCGN1761ENX. pCIB1004 is cleaved with *NcoI* and the resultant 3' overhang of the linearized fragment is rendered blunt by treatment with T4 DNA polymerase. The fragment is then cleaved with *HindIII* and the resultant PR-1a promoter containing fragment is gel purified and cloned into pCGN1761ENX from which the double 35S promoter has been removed. This is done by cleavage with *XhoI* and blunting with T4 polymerase, followed by cleavage with *HindIII* and isolation of the larger vector-terminator containing fragment into which the pCIB1004 promoter fragment is cloned. This generates a pCGN1761ENX derivative with the PR-1a promoter and the *tml* terminator and an intervening polylinker with unique *EcoRI* and *NotI* sites.

A nucleotide sequence encoding a DNA molecule of the present invention is inserted into this vector, and the fusion product (*i.e.* promoter-gene-terminator) is subsequently transferred to any selected transformation vector, including those described in this application, thus providing for chemically inducible expression of the transactivator.

(2) Ethanol-Inducible Promoter

A promoter inducible by certain alcohols or ketones, such as ethanol, is also used to confer inducible expression of a transgene of the present invention. Such a promoter is for example the *alcA* gene promoter from *Aspergillus nidulans* (Caddick et al. (1998) Nat Biotechnol 16:177-180). In *A. nidulans*, the *alcA* gene encodes alcohol dehydrogenase I, whose expression is regulated by the AlcR transcription factors in presence of the chemical inducer. For the purposes of the present invention, the CAT gene sequences in plasmid palcA:CAT comprising a *alcA* gene promoter sequence fused to a minimal 35S promoter (Caddick et al. (1998) Nat Biotechnol 16:177-180) are replaced by a nucleotide sequence encoding a transgene to form an expression cassette having the nucleotide sequence encoding a transgene under the control of the *alcA* gene promoter. This is carried out using methods well known in the art. In a preferred embodiment, the minimal 35S promoter is replaced by any convenient minimal promoter, such as e.g. the maize Bronze-1 minimal promoter (Roth et al. (1991) The Plant Cell 3: 317-325). Termination signals in palcA:CAT can also be replaced by other termination signals well known in the art. The resulting construct is transformed into a desired plant species. The *alcR* gene is also comprised in the plant comprising the nucleotide sequence encoding a DNA molecule of the present invention fused to the *alcA* gene promoter and the expression of the *alcR* gene is controlled

by any promoter suitable for expression in plants known in the art or described here. Thus, tissue- or organ-specificity of the *alcR* gene product is achieved leading to inducible tissue- or organ-specificity of the transgene. Any termination signals known in the art are also suitable for the *alcR* expression cassette.

(3) Glucocorticoid-Inducible Promoter

Induction of expression of a DNA molecule of the present invention using systems based on steroid hormones is also contemplated. For example, a glucocorticoid-mediated induction system is used (Aoyama and Chua (1997) The Plant Journal 11: 605-612) and gene expression is induced by application of a glucocorticoid, for example a synthetic glucocorticoid, preferably dexamethasone, preferably at a concentration ranging from 0.1 mM to 1 mM, more preferably from 10 mM to 100 mM. For the purposes of the present invention, the luciferase gene sequences are replaced by a nucleotide sequence encoding a transgene to form an expression cassette having the nucleotide sequence encoding a transgene under the control of six copies of the GAL4 upstream activating sequences fused to the 35S minimal promoter. This is carried out using methods well known in the art. In a preferred embodiment, the minimal 35S promoter is replaced by any convenient minimal promoter, such as, e.g., the maize Bronze-1 minimal promoter (Roth et al. (1991) The Plant Cell 3: 317-325). Termination signals can also be replaced by other termination signals well known in the art. The resulting construct is transformed into a desired plant species. The trans-acting factor comprises the GAL4 DNA-binding domain (Keegan et al. (1986) Science 231: 699-704) fused to the transactivating domain of the herpes viral protein VP16 (Triezenberg et al. (1988) Genes Devel. 2: 718-729) fused to the hormone-binding domain of the rat glucocorticoid receptor (Picard et al. (1988) Cell 54: 1073-1080). The expression of the fusion protein is controlled by any promoter suitable for expression in plants known in the art or described here. This expression cassette is also comprised in the plant comprising the nucleotide sequence encoding a transgene fused to the 6xGAL4/minimal promoter. Thus, tissue- or organ-specificity of the fusion protein is achieved leading to inducible tissue- or organ-specificity of the transgene. Any termination signals known in the art are also suitable for the expression cassette comprising the fusion protein.

Constitutive Expression: the Actin Promoter

Several isoforms of actin are known to be expressed in most cell types and consequently the actin promoter is a good choice for a constitutive promoter. In particular, the promoter

from the rice *Act1* gene has been cloned and characterized (McElroy *et al.* Plant Cell 2: 163-171 (1990)). A 1.3 kb fragment of the promoter is found to contain all the regulatory elements required for expression in rice protoplasts. Furthermore, numerous expression vectors based on the *Act1* promoter have been constructed specifically for use in monocotyledons (McElroy *et al.* Mol. Gen. Genet. 231: 150-160 (1991)). These incorporate the *Act1*-intron 1, *Adh1* 5' flanking sequence and *Adh1*-intron 1 (from the maize alcohol dehydrogenase gene) and sequence from the CaMV 35S promoter. Vectors showing highest expression are fusions of 35S and the *Act1* intron or the *Act1* 5' flanking sequence and the *Act1* intron. Optimization of sequences around the initiating ATG (of the GUS reporter gene) also enhanced expression. The promoter expression cassettes described by McElroy *et al.* (Mol. Gen. Genet. 231: 150-160 (1991)) can be easily modified for the expression of the nucleotide sequence of the present invention and are particularly suitable for use in monocotyledonous hosts. For example, promoter containing fragments can be removed from the McElroy constructions and used to replace the double 35S promoter in pCGN1761ENX, which is then available for the insertion or specific gene sequences. The fusion genes thus constructed can then be transferred to appropriate transformation vectors. In a separate report the rice *Act1* promoter with its first intron has also been found to direct high expression in cultured barley cells (Chibbar *et al.* Plant Cell Rep. 12: 506-509 (1993)).

A nucleotide sequence encoding a DNA molecule of the present invention is inserted downstream of such a promoter, and the fusion products (*i.e.* promoter-gene-terminator) are subsequently transferred to any selected transformation vector, including those described in this application.

Constitutive Expression: the Ubiquitin Promoter

Ubiquitin is another gene product known to accumulate in many cell types and its promoter has been cloned from several species for use in transgenic plants (*e.g.* sunflower - Binet *et al.* Plant Science 79: 87-94 (1991), maize - Christensen *et al.* Plant Molec. Biol. 12: 619-632 (1989)). The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence and vectors constructed for monocot transformation are disclosed in the patent publication EP 0 342 926. Further, Taylor *et al.* (Plant Cell Rep. 12: 491-495 (1993)) describe a vector (pAHC25) which comprises the maize ubiquitin promoter and first intron and its high activity in cell suspensions of numerous monocotyledons when introduced via microprojectile bombardment. The ubiquitin promoter is clearly suitable for the expression of

the nucleotide sequence in transgenic plants, especially monocotyledons. Suitable vectors are derivatives of pAHC25 or any of the transformation vectors described in this application, modified by the introduction of the appropriate ubiquitin promoter and/or intron sequences.

The Arabidopsis Ubiquitin 3 (UBQ3) gene promoter (Norris et al. (1993*)* Plant Mol Biol 21:895-906) is also a promoter suitable for expression of a transgene of the present invention in plants.

A nucleotide sequence encoding a transgene is therefore inserted into any of these vectors, and the fusion products (*i.e.* promoter-gene-terminator) are used for transformation of plants, resulting in constitutive expression of the transgene.

Root Specific Expression

A preferred pattern of expression for a transgene of the present invention is root expression. A suitable root promoter is that described by de Framond (FEBS 290: 103-106 (1991)) and also in the published patent application EP 0 452 269. This promoter is transferred to a suitable vector such as pCGN1761ENX and a nucleotide sequence encoding a transgene is inserted into such vector. The entire promoter-gene-terminator cassette is subsequently transferred to a transformation vector of interest.

Wound Inducible Promoters

Wound-inducible promoters are particularly suitable for the expression of a transgene. Numerous such promoters have been described (*e.g.* Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), Warner *et al.* Plant J. 3: 191-201 (1993)) and all are suitable for use with the instant invention. Logemann *et al.* (*supra*) describe the 5' upstream sequences of the dicotyledonous potato *wun1* gene. Xu *et al.* (*supra*) show that a wound inducible promoter from the dicotyledon potato (*pin2*) is active in the monocotyledon rice. Further, Rohrmeier & Lehle (*supra*) describe the cloning of the maize *Wip1* cDNA which is wound induced and which can be used to isolated the cognate promoter using standard techniques. Similarly, Firek *et al.* (*supra*) and Warner *et al.* (*supra*) have described a wound induced gene from the monocotyledon *Asparagus officinalis* which is expressed at local wound and pathogen invasion sites. Using cloning techniques well known in the art, these promoters can be transferred to suitable vectors, fused to a nucleotide sequence encoding a transgene, and used to express these genes at the sites of insect pest infection.

Pith Preferred Expression

Patent application WO 93/07278 describes the isolation of the maize *trpA* gene which is preferentially expressed in pith cells. Using standard molecular biological techniques, this promoter or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a transgene of the present invention in a pith-preferred manner. In fact fragments containing the pith-preferred promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants. Pith preferred expression of a nucleotide sequence encoding a transgene is achieved by inserting the nucleotide sequence encoding a transgene in such vector.

Pollen-Specific Expression

Patent Application WO 93/07278 further describes the isolation of the maize calcium-dependent protein kinase (CDPK) gene which is expressed in pollen cells. The gene sequence and promoter extend up to 1400 bp from the start of transcription. Using standard molecular biological techniques, this promoter or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a transgene of the present invention in a pollen-specific manner. In fact fragments containing the pollen-specific promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants.

Leaf-Specific Expression

A maize gene encoding phosphoenol carboxylase (PEPC) has been described by Hudspeth & Grula (Plant Molec Biol 12: 579-589 (1989)). Using standard molecular biological techniques the promoter for this gene is used to drive the expression of a transgene of the present invention in a leaf-specific manner in transgenic plants.

Expression with Chloroplast Targeting

Chen & Jagendorf (J. Biol. Chem. 268: 2363-2367 (1993) have described the successful use of a chloroplast transit peptide for import of a heterologous transgene. This peptide used is the transit peptide from the *rbcS* gene from *Nicotiana plumbaginifolia* (Poulsen *et al.* Mol. Gen. Genet. 205: 193-200 (1986)). Using the restriction enzymes *DraI* and *SphI*, or *Tsp509I* and *SphI* the DNA sequence encoding this transit peptide can be excised from plasmid prbcS-8B (Poulsen *et al. supra*) and manipulated for use with any of the constructions described above. The *DraI-SphI* fragment extends from -58 relative to the initiating *rbcS* ATG to, and including, the first amino acid (also a methionine) of the mature peptide immediately after the import cleavage site, whereas the *Tsp509I-SphI* fragment extends from -8 relative to the initiating *rbcS* ATG to, and including, the first amino acid of

the mature peptide. Thus, these fragment can be appropriately inserted into the polylinker of any chosen expression cassette generating a transcriptional fusion to the untranslated leader of the chosen promoter (*e.g.* 35S, PR-1a, actin, ubiquitin *etc.*), whilst enabling the insertion of a nucleotide sequence encoding a transgene in correct fusion downstream of the transit peptide. Constructions of this kind are routine in the art. For example, whereas the *DraI* end is already blunt, the 5' *Tsp509I* site may be rendered blunt by T4 polymerase treatment, or may alternatively be ligated to a linker or adaptor sequence to facilitate its fusion to the chosen promoter. The 3' *SphI* site may be maintained as such, or may alternatively be ligated to adaptor of linker sequences to facilitate its insertion into the chosen vector in such a way as to make available appropriate restriction sites for the subsequent insertion the nucleotide sequence encoding a transactivator. Ideally the ATG of the *SphI* site is maintained and comprises the first ATG of the DNA molecule. Chen & Jagendorf (*supra*) provide consensus sequences for ideal cleavage for chloroplast import, and in each case a methionine is preferred at the first position of the mature protein. At subsequent positions there is more variation and the amino acid may not be so critical. In any case, fusion constructions can be assessed for efficiency of import *in vitro* using the methods described by Bartlett *et al.* (In: Edelmann *et al.* (Eds.) Methods in Chloroplast Molecular Biology, Elsevier. pp 1081-1091 (1982)) and Wasmann *et al.* (Mol. Gen. Genet. 205: 446-453 (1986)). Typically the best approach may be to generate fusions using the DNA molecule with no modifications at the aminoterminus, and only to incorporate modifications when it is apparent that such fusions are not chloroplast imported at high efficiency, in which case modifications may be made in accordance with the established literature (Chen & Jagendorf, *supra*; Wasman *et al., supra*; Ko & Ko, J. Biol. Chem. 267: 13910-13916 (1992)).

A preferred vector is constructed by transferring the *DraI-SphI* transit peptide encoding fragment from prbcS-8B to the cloning vector pCGN1761ENX/Sph-. This plasmid is cleaved with *EcoRI* and the termini rendered blunt by treatment with T4 DNA polymerase. Plasmid prbcS-8B is cleaved with *SphI* and ligated to annealed molecular adaptors. The resultant product is 5'-terminally phosphorylated by treatment with T4 kinase. Subsequent cleavage with *DraI* releases the transit peptide encoding fragment which is ligated into the blunt-end ex-*EcoRI* sites of the modified vector described above. Clones oriented with the 5' end of the insert adjacent to the 3' end of the 35S promoter are identified by sequencing. These clones carry a DNA fusion of the 35S leader sequence to the *rbcS-8A* promoter-transit

peptide sequence extending from -58 relative to the *rbcS* ATG to the ATG of the mature protein, and including at that position a unique *SphI* site, and a newly created *EcoRI* site, as well as the existing *NotI* and *XhoI* sites of pCGN1761ENX. This new vector is designated pCGN1761/CT. DNA sequences are transferred to pCGN1761/CT in frame by amplification using PCR techniques and incorporation of an *SphI, NsphI,* or *NlaIII* site at the amplified ATG, which following restriction enzyme cleavage with the appropriate enzyme is ligated into *SphI*-cleaved pCGN1761/CT. To facilitate construction, it may be required to change the second amino acid of cloned gene, however, in almost all cases the use of PCR together with standard site directed mutagenesis will enable the construction of any desired sequence around the cleavage site and first methionine of the mature protein.
A further preferred vector is constructed by replacing the double 35S promoter of pCGN1761ENX with the *BamHI-SphI* fragment of prbcS-8A which contains the full-length light regulated *rbcS-8A* promoter from -1038 (relative to the transcriptional start site) up to the first methionine of the mature protein. The modified pCGN1761 with the destroyed *SphI* site is cleaved with *PstI* and *EcoRI* and treated with T4 DNA polymerase to render termini blunt. prbcS-8A is cleaved *SphI* and ligated to the annealed molecular adaptor of the sequence described above. The resultant product is 5'-terminally phosphorylated by treatment with T4 kinase. Subsequent cleavage with *BamHI* releases the promoter-transit peptide containing fragment which is treated with T4 DNA polymerase to render the *BamHI* terminus blunt. The promoter-transit peptide fragment thus generated is cloned into the prepared pCGN1761ENX vector, generating a construction comprising the *rbcS-8A* promoter and transit peptide with an *SphI* site located at the cleavage site for insertion of heterologous genes. Further, downstream of the *SphI* site there are *EcoRI* (re-created), *NotI,* and *XhoI* cloning sites. This construction is designated pCGN1761rbcS/CT.
Similar manipulations can be undertaken to utilize other GS2 chloroplast transit peptide encoding sequences from other sources (monocotyledonous and dicotyledonous) and from other genes.

Modification of pCGN1761ENX by Optimization of the Translational Initiation Site

For any of the constructions described in this section, modifications around the cloning sites can be made by the introduction of sequences which may enhance translation. This is particularly useful when genes derived from microorganisms are to be introduced into plant expression cassettes as these genes may not contain sequences adjacent to their initiating

methionine which may be suitable for the initiation of translation in plants. In cases where genes derived from microorganisms are to be cloned into plant expression cassettes at their ATG it may be useful to modify the site of their insertion to optimize their expression. Modification of pCGN1761ENX is described by way of example to incorporate one of several optimized sequences for plant expression (*e.g.* Joshi, *supra*).
pCGN1761ENX is cleaved with *SphI*, treated with T4 DNA polymerase and religated, thus destroying the *SphI* site located 5' to the double 35S promoter. This generates vector pCGN1761ENX/Sph-. pCGN1761ENX/Sph- is cleaved with *EcoRI*, and ligated to an annealed molecular adaptor. This generates the vector pCGNSENX which incorporates the *quasi*-optimized plant translational initiation sequence TAAA-C adjacent to the ATG which is itself part of an *SphI* site which is suitable for cloning heterologous genes at their initiating methionine. Downstream of the *SphI* site, the *EcoRI, NotI,* and *XhoI* sites are retained.
An alternative vector is constructed which utilizes an *NcoI* site at the initiating ATG. This vector, designated pCGN1761NENX is made by inserting an annealed molecular adaptor at the pCGN1761ENX *EcoRI* site. Thus, the vector includes the *quasi*-optimized sequence TAAACC adjacent to the initiating ATG which is within the *NcoI* site. Downstream sites are *EcoRI, NotI,* and *XhoI.* Prior to this manipulation, however, the two *NcoI* sites in the pCGN1761ENX vector (at upstream positions of the 5' 35S promoter unit) are destroyed using similar techniques to those described above for *SphI* or alternatively using "inside-outside" PCR (Innes *et al.* PCR Protocols: A guide to methods and applications. Academic Press, New York (1990)). This manipulation can be assayed for any possible detrimental effect on expression by insertion of any plant cDNA or reporter gene sequence into the cloning site followed by routine expression analysis in plants.
Accordingly, a nucleotide sequence encoding a transgene is inserted into pCGN1761ENX for constitutive expression under the control of the CaMV 35S promoter with an optimized Translation Initiation Site.

Example 34: Construction of Plant Transformation Vectors

Numerous transformation vectors are available for plant transformation, and a nucleotide sequence encoding a DNA molecule of the present invention is inserted into any of the expression cassettes described above, such that they are capable of expressing the transgene in desirable cells, under appropriate conditions. A resulting expression cassette is then incorporated into any appropriate transformation vector described below.

The selection of vector for use will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene which confers resistance to kanamycin and related antibiotics (Vieira and Messing, Gene 19: 259-268 (1982); Bevan *et al.*, Nature 304:184-187 (1983)), the *hph* gene which confers resistance to the antibiotic hygromycin (Blochlinger & Diggelmann, Mol Cell Biol 4: 2929-2931), the *dhfr* gene, which confers resistance to methatrexate (Bourouis and Jarry., EMBO J. 2(7): 1099-1104 (1983)), and the bacterial *aad*A gene (Goldschmidt-Clermont, 1991, Nucl. Acids Res. 19: 4083-4089), encoding aminoglycoside 3'-adenylyltransferase and conferring resistance to streptomycin or spectinomycin. Other markers to be used include the *bar* gene which confers resistance to the herbicide phosphinothricin (White *et al.*, Nucl Acids Res 18: 1062 (1990), Spencer *et al.* Theor Appl Genet 79: 625-631 (1990)), a mutant EPSP synthase gene encoding glyphosate resistance (Hinchee et al., 1988, Bio/Technology 6: 915-922), a mutant acetolactate synthase (ALS) gene which confers imidazolione or sulfonylurea resistance (Lee et al., 1988, EMBO J. 7: 1241-1248), a mutant *psbA* gene conferring resistance to atrazine (Smeda et al., 1993, Plant Physiol. 103: 911-917), or a mutant protoporphyrinogen oxidase gene as described in EP 0 769 059.
Selection markers resulting in positive selection, such as a phosphomannose isomerase gene, as described in patent application WO 93/05163, are also used.
Identification of transformed cells may also be accomplished through expression of screenable marker genes such as genes coding for chloramphenicol acetyl transferase (CAT), β-glucuronidase (GUS), luciferase, and green fluorescent protein (GFP) or any other protein that confers a phenotypically distinct trait to the transformed cell.

(1) Construction of Vectors Suitable for *Agrobacterium* Transformation
Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). Below the construction of two typical vectors is described.

Construction of pCIB200 and pCIB2001

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and is constructed in the following manner. pTJS75kan

is created by *NarI* digestion of pTJS75 (Schmidhauser & Helinski, J Bacteriol. 164: 446-455 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII (Vieira and Messing, Gene 19: 259-268 (1982); Bevan *et al.*, Nature 304: 184-187 (1983); McBride *et al.*, Plant Molecular Biology 14: 266-276 (1990)). *XhoI* linkers are ligated to the *EcoRV* fragment of pCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein *et al.*, Gene 53: 153-161 (1987)), and the *XhoI*-digested fragment is cloned into *SalI*-digested pTJS75kan to create pCIB200 (see also EP 0 332 104). pCIB200 contains the following unique polylinker restriction sites: *EcoRI, SstI, KpnI, BglII, XbaI,* and *SalI. pCIB2001* is a derivative of pCIB200 which created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI, SstI, KpnI, BglII, XbaI, SalI, MluI, BclI, AvrII, ApaI, HpaI,* and *StuI.* pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals. Any one of the plant expression cassettes described above is inserted into pCIB2001, preferably using the polylinker.

Construction of pCIB10 and Hygromycin Selection Derivatives thereof

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants, T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium.* Its construction is described by Rothstein *et al.* (Gene 53: 153-161 (1987)). Various derivatives of pCIB10 have been constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz *et al.* (Gene 25: 179-188 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717). This vectors is used transform an expression cassette of the present invention.

(2) Construction of Vectors Suitable for non-*Agrobacterium* Transformation.

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking

these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques which do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (*e.g.* PEG and electroporation), microinjection or pollen transformation (US Patent 5,629,183). The choice of vector depends largely on the preferred selection for the species being transformed. Below, the construction of some typical vectors is described.

Construction of pCIB3064

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide Basta (or phosphinothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in the PCT published application WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites are mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites *SspI* and *PvuII.* The new restriction sites are 96 and 37 bp away from the unique *SalI* site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 is designated pCIB3025. The GUS gene is then excised from pCIB3025 by digestion with *SalI* and *SacI*, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 is obtained from the John Innes Centre, Norwich and the a 400 bp *SmaI* fragment containing the *bar* gene from *Streptomyces viridochromogenes* is excised and inserted into the *HpaI* site of pCIB3060 (Thompson *et al.* EMBO J 6: 2519-2523 (1987)). This generated pCIB3064 which comprises the *bar* gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene for ampicillin resistance (for selection in *E. coli*) and a polylinker with the unique sites *SphI, PstI, HindIII,* and *BamHI*. This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals to direct expression of a transgene of the present invention.

Construction of pSOG19 and pSOG35

pSOG35 is a transformation vector which utilizes the *E. coli* gene dihydrofolate reductase (DHFR) as a selectable marker conferring resistance to methotrexate. PCR is used to amplify the 35S promoter (~800 bp), intron 6 from the maize Adh1 gene (~550 bp) and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250 bp fragment encoding the *E. coli* dihydrofolate reductase type II gene is also amplified by PCR and these two PCR fragments are assembled with a *SacI-PstI* fragment from pBI221 (Clontech) which

comprised the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generated pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generated the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have *HindIII, SphI, PstI* and *EcoRI* sites available for the cloning of foreign sequences, in particular a transgene of the present invention.

Plastid Transformation Vectors

For constitutive expression of the nucleotide sequence of the present invention in plant plastids under control of the clpP gene promoter elements, plastid transformation vector pPH143 (example 36 of WO 97/32011) is used. The nucleotide sequence is inserted into pPH143 thereby replacing the PROTOX coding sequence. This vector is then used for plastid transformation and selection of transformants for spectinomycin resistance. Alternatively, the nucleotide sequence is inserted in pPH143 so that it replaces the *aadA* gene under control of the *psbA* gene promoter. In this case, transformants are selected for resistance to PROTOX inhibitors. In an alternative embodiment, promoters from the plastid 16S ribosomal RNA genes of tobacco or Arabidopsis are used to express the PROTOX coding sequence.

Example 35: Plastid Transformation of Maize

Type I embryogenic callus cultures (Green et al. (1983) in A. Fazelahmad, K. Downey, J. Schultz, R.W. Voellmy, eds. Advances in Gene Technology: Molecular Genetics of Plants and Animals. Miami Winter Symposium Series, Vol. 20. Academic Press, N.Y.) are initiated from immature maize embryos, 1.5 - 2.5 mm in length, from greenhouse grown material. Embryos are aseptically excised from surface-sterilized ears approximately 14 days after pollination. Embryos are either placed on D callus initiation media with 2% sucrose and 5 mg/L chloramben (Duncan et al. (1985) Planta 165: 322-332) or onto KM callus initiation media with 3% sucrose and 0.75 mg/L 2,4-d (Kao and Michayluk (1975) Planta 126, 105-110). Embryos and embryogenic cultures are subsequently cultured in the dark. Embryogenic responses are removed from the explants after ~14 days. Embryogenic responses from D callus initiation media are placed onto D callus maintenance media with 2% sucrose and 0.5 mg/L 2,4-d while those of while those from KM callus initiation media

are placed onto KM callus maintenance media with 2% sucrose and 5 mg/L Dicamba. After 3 to 8 weeks of weekly selective subculture to fresh maintenance media, high quality compact embryogenic cultures are established. Actively growing embryogenic callus pieces are selected as target tissue for gene delivery. The callus pieces are plated onto target plates containing maintenance medium with 12% sucrose approximately 4 hours prior to gene delivery. The callus pieces are arranged in circles, with radii of 8 and 10 mm from the center of the target plate. Plasmid DNA is precipitated onto gold microcarriers as described in the DuPont Biolistics manual. Two to three μg of each plasmid is used in each 6 shot microcarrier preparation. Genes are delivered to the target tissue cells using the PDS-1000He Biolistics device. The settings on the Biolistics device are as follows: 8 mm between the rupture disc and the macrocarrier, 10 mm between the macrocarrier and the stopping screen and 7 cm between the stopping screen and the target. Each target plate is shot twice using 650 psi rupture discs. A 200 X 200 stainless steel mesh (McMaster-Carr, New Brunswick, NJ) is placed between the stopping screen and the target tissue.
Five days later, the bombed callus pieces are transferred to maintenance medium with 2% sucrose and 0.5 mg/L 2,4-d, but without amino acids, and containing 750 or 1000 nM Formula XVII. The callus pieces are placed for 1 hour on the light shelf 4-5 hours after transfer or on the next day, and stored in the dark at 27°C for 5-6 weeks. Following the 5-6 week primary selection stage, yellow to white tissue is transferred to fresh plates containing the same medium supplemented with 500 or 750 nM Formula XVII. 4-5 hours after transfer or on the next day, the tissues are placed for 1 hour on the light shelf and stored in the dark at 27°C for 3-4 weeks. Following the 3-4 week secondary selection stage, the tissues are transferred to plates containing the same medium supplemented with 500 nM Formula XVII. Healthy growing tissue is placed for 1 hour on the light shelf and stored in the dark at 27°C. It is subcultured every two weeks until the colonies are large enough for regeneration.
At that point, colonies are transferred to a modified MS medium (Murashige and Skoog (1962) Physiol. Plant 15: 473-497) containing 3% sucrose (MS3S) with no selection agent and placed in the light. Either 0.25 mg/L ancymidol and 0.5 mg/L kinetin are added to this medium to induce embryo germination or 2 mg/L benzyl adenine is added. Regenerating colonies are transferred to MS3S media without ancymidol and kinetin, or benzyl adenine, respectively, after 2 weeks. Regenerating shoots with or without roots are transferred to boxes containing MS3S medium and small plants with roots are eventually recovered and transferred to soil in the greenhouse.

Example 36: Preparation of a chimeric gene containing the ragweed pollen allergen *Amb a I.1* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The ragweed pollen allergen *Amb a I.1* coding sequence is fused to the *Arabidopsis* UBQ3 promoter (Norris et al. (1993) Plant Mol. Biol. 21: 895-906) by inserting into the *Nco*I, *Xba*I sites of pPH121 the 5' half of *Amb a I.1* as a 0.83 kb *Nco*I, *Sal*I fragment from pAT230 and the 3' end of *Amb a I.1* as a 0.39 kb *Sal*I, *Xba*I PCR generated fragment designed to remove the dam methylated *Xba*I site at the 3' end of the *Amb a I.1* gene. PCR amplification is performed using pAT230 as a template and the following primer pair: the "top strand" primer (5'-GTC GCT TTC AAC ACG TTC AC-3', SEQ ID NO:31) and the "bottom strand" primer which removes the A before the *Xba*I site (5'-GCG CTC TAG ACA TTA TAA GTG CTT AGT-3', SEQ ID NO:32). The 452 bp amplification product is gel purified, digested with *Sal*I and *Xba*I and ligated as above, producing pAT240. The *Hind*III-*Xba*I of pAT240 containing the UBQ3 promoter driving the *Amb a I.1* gene is ligated into a 11.3 kb *Hind*III, *Xba*I digested pPH108 fragment, producing a binary vector carrying a constitutively expressed *Amb a I.1* gene.

Example 37: Preparation of a chimeric gene containing the putative mature ragweed allergen *Amb a I.1* coding sequence fused to the constitutive Arabidopsis UBQ3 promoter for nuclear expression in plants

The *Amb a I.1* has a putative signal peptide cleavage site, with the alanine at position 26 predicted to be residue +1 (Rafnar et al. (1991) J. Biol. Chem. 266: 1229-1236). The *Amb a I.1* coding sequence is modified to remove the signal peptide by PCR amplification using pAT230 as a template and the following oligonucleotide pair: the "top strand" primer places an ATG before the first codon of the mature *Amb a I.1* protein, the first 20 bp of the mature protein and a *NcoI* site at the newly created ATG (5'-GCA CCA TGG CCG AAG ATC TCC AGG AAA T-3', SEQ ID NO:33) and the "bottom strand" primer (5'-CTA CCA GCC CAT CAA CAG ACT TAC-3', SEQ ID NO:34). A 594 bp amplification product is produced comprised of the 5' end of the *Amb a I.1* gene without the signal sequence. The fragment is gel purified, digested with *NcoI* and *ClaI* and the 0.35 kb fragment is ligated with the 3' end of the *Amb a I.1* gene as a 0.81 kb *ClaI*, *XbaI* fragment into the *NcoI*, *XbaI* 4.4 kb fragment of pAT240, creating a *Amb a I.1* gene without the signal peptide fused to the

Arabidopsis UBQ3 promoter, which is cloned along with a terminator sequence into a binary vector.

Example 38: Preparation of a chimeric gene containing the *Dermatophagoides farinae* major allergen *Der f I* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The Dermatophagoides allergen *Der f I* coding sequence cloned as described in example 13 is fused to the *Arabidopsis* UBQ3 promoter and inserted into a binary vector as described in example 34.

Example 39: Preparation of a chimeric gene containing the *Dermatophagoides farinae* major allergen *Der f II* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The *Dermatophagoides* allergen *Der f II* coding sequence cloned as described in example 13 is fused to the *Arabidopsis* UBQ3 promoter and inserted into a binary vector as described in example 34.

Example 40: Preparation of a chimeric gene containing the *Dermatophagoides pteronyssinus* major allergen *Der p I* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The *Dermatophagoides* allergen *Der p I* coding sequence cloned as described in example 13 is fused to the *Arabidopsis* UBQ3 promoter and inserted into a binary vector as described in example 34.

Example 41: Preparation of a chimeric gene containing the *Dermatophagoides pteronyssinus* major allergen *Der p II* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The *Dermatophagoides* allergen *Der p II* coding sequence cloned as described in example 13 is fused to the *Arabidopsis* UBQ3 promoter and inserted into a binary vector as described in example 34.

Example 42: Preparation of a chimeric gene containing the Johnson grass allergen *Sor h I* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The Johnson grass allergen *Sor h I* coding sequence is fused to the *Arabidopsis* UBQ3 promoter and inserted into a binary vector as described in example 34.

Example 43: Preparation of a chimeric gene containing the birch pollen allergen *Bet V I* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The birch pollen allergen *Bet V I* coding sequence is fused to the *Arabidopsis* UBQ3 promoter and inserted into a binary vector as described in example 34.

Example 44: Preparation of a chimeric gene containing the mosquito salivary allergen *rAed a 1* coding sequence fused to the constitutive *Arabidopsis* UBQ3 promoter for nuclear expression in plants

The *rAed a 1* coding sequence is fused to the *Arabidopsis* UBQ3 promoter and inserted into a binary vector as described in example 34.

Example 45: Preparation of chimeric genes for vacuolar expression containing the ragweed pollen allergen *Amb a I.1* coding sequence fused to the constitutive Arabidopsis UBQ3 promoter

Plasmid pAT240 containing the ragweed pollen allergen gene *Amb a I.1* is used as a template for PCR with a "top strand" primer extending from position 775 to 799 in the *Amb a I.1* gene relative to the endogenous ATG (5'-GCA ACG GTC GCT TTC AAC ACG TTC A-3', SEQ ID NO:35) and a "bottom strand" primer whose sequence is homologous to the last 21 bp of the *Amb a I.1* and includes 21 bp of a vacuolar targeting sequence derived from a tobacco chitinase gene (Shinshi et al., (1990) Plant Mol. Biol. 14, 357-368, Neuhaus et al. (1991) Proc. Natl. Acad. Sci. USA 88, 10362-10366), the last codon of the same tobacco chitinase gene and a *XbaI* restriction site (5'-CGC TCT AGA TTA CAT AGT ATC GAC TAA AAG TCC GCA AGG TGC TCC GGG TTG GCA-3', SEQ ID NO:36). PCR amplification generates a 447 bp product which is gel purified and digested with *SalI* and *XbaI*. The 383 bp *SalI*, *XbaI* fragment containing the 3' end of *Amb a I.1* fused to a tobacco chitinase vacuolar targeting sequence is ligated to the 5' end of *Amb a I.1* as a 0.83 kb *NcoI*, *SalI*

fragment from pAT240 into the 4.4 kb *NcoI*, *XbaI* fragment of pAT240 containing the UBQ3 promoter and vector. The Arabidopsis UBQ3 promoter :: *Amb a I.1* coding sequence fused to a tobacco chitinase vacuolar targeting sequence cassette is cloned with a terminator sequence into a binary vector.

Plasmid pSCH10 (Shinshi et al., (1990) Plant Mol. Biol. 14, 357-368, Neuhaus et al. (1991) Proc. Natl. Acad. Sci. USA 88, 10362-10366) is used as a template for PCR amplification with a "top strand" primer whose sequence is homologous to the first 22 bp of the tobacco chitinase 23 amino acid N-terminal signal peptide and a *BspHI* restriction site at the ATG (ErspU 5'-CGG TCA TGA GGC TTT GTA AAT TCA CAG-3', SEQ ID NO:37) and a "bottom strand" primer whose sequence is homologous to the last 17 bp of the tobacco chitinase N-terminal signal peptide fused to the first 14 bp of the putative mature 5' end of the *Amb a I.1* gene (ErspL 5'-TGG AGA TCT TCG GCT GCC GAG GCA GAA AGC A-3', SEQ ID NO:38). The 88 bp amplification product is gel purified, cleaved with *BspHI* and *BglII* and the 76 bp fragment containing the tobacco chitinase 23 amino acid N-terminal signal peptide fused to the 5' end of the *Amb a I.1* gene without the native signal peptide is ligated to the 3' end of the *Amb a I.1* gene containing the tobacco chitinase vacuolar targeting sequence as a *BglII*, *XbaI* fragment into the 4.4 kb *NcoI*, *XbaI* fragment of pAT240 containing the UBQ3 promoter and vector. The Arabidopsis UBQ3 promoter :: *Amb a I.1* gene with the native signal peptide removed, a N-terminal tobacco chitinase signal peptide and a C-terminal tobacco chitinase vacuolar targeting sequence cassette is cloned with a terminator sequence into a binary vector.

Plasmid pSCH10 is used as a template for PCR amplification with a "top strand" primer whose sequence is homologous to the first 22 bp of the tobacco chitinase 23 amino acid N-terminal signal peptide and a *BspHI* restriction site at the ATG (ErspU) and a "bottom strand" primer whose sequence is homologous to the last 17 bp of the tobacco chitinase N-terminal signal peptide fused to a 5' extension of the first 13 bp of *Amb a I.1* (ErspovL 5'-AGT GTT TGA TCC CTG CCG AGG CAG AAA GCA-3', SEQ ID NO:39). Plasmid pAT240 is used as a template for PCR amplification with a "top strand" primer whose sequence is homologous to the first 25 bp after the start codon of *Amb a I.1* and which anneals to 13 bp of primer ErspovL (AmboeU 5'-GGG ATC AAA CAC TGT TGT TAC ATC T-3', SEQ ID NO:40) and a "bottom strand" primer extending from positions 135 to 158 in the *Amb a I.1* gene relative to the endogenous ATG. The two PCR amplification products are gel purified and the PCR overlap extension technique is performed to fuse the two products together as

follows: 2 ml of each purified amplification product is combined in one PCR reaction tube and 10 cycles are done in the absence of primers in order to anneal and extend the fusion product, then 5 ml of the reaction is used as a template for PCR amplification using the "outside" primers ErspU and AmboeL. The final amplification product consists of the tobacco chitinase N-terminal 23 amino acid signal peptide with a *BspHI* site at the initiating codon fused in frame to the *Amb a I.1* gene from the first amino acid after the ATG to 155 bp downstream. The product is gel purified, digested with *BspHI* and *BglII* and ligated to the 3' end of the *Amb a I.1* gene containing the tobacco chitinase vacuolar targeting sequence as a *BglII*, *XbaI* fragment into the 4.4 kb *NcoI*, *XbaI* fragment of pAT240 containing the UBQ3 promoter and vector. The Arabidopsis UBQ3 promoter :: *Amb a I.1* gene , a N-terminal tobacco chitinase signal peptide and a C-terminal tobacco chitinase vacuolar targeting sequence cassette is cloned with a terminator sequence into a binary vector.

Example 46: Preparation of chimeric genes for vacuolar expression containing the ragweed pollen allergen *Amb a I.1* coding sequence fused to the tobacco PR-1a promoter

The PR-1a promoter from PR-1aDXhoNco (Uknes et al. (1993), The Plant Cell 5, 159-169) is excised as a 903 bp *XhoI*, *NcoI* fragment and ligated to a *NcoI*, *XbaI* fragment containing the *Amb a I.1* gene, a N-terminal tobacco chitinase signal peptide and a C-terminal tobacco chitinase vacuolar targeting sequence into the *XhoI*, *XbaI* sites of pLITMUS28 (New England Biolabs). The PR-1a promoter :: *Amb a I.1* gene, a N-terminal tobacco chitinase signal peptide and a C-terminal tobacco chitinase vacuolar targeting sequence cassette is cloned with a terminator sequence into a binary vector. The *XhoI*, *NcoI* PR-1a promoter fragment is also ligated to a *NcoI*, *XbaI* fragment containing the *Amb a I.1* gene with the native signal peptide removed, a N-terminal tobacco chitinase signal peptide and a C-terminal tobacco chitinase vacuolar targeting sequence cassette into the *XhoI*, *XbaI* sites of pLITMUS28. The PR-1a promoter :: *Amb a I.1* gene with the native signal peptide removed, a N-terminal tobacco chitinase signal peptide and a C-terminal tobacco chitinase vacuolar targeting sequence cassette is cloned with a terminator sequence into a binary vector. Upon reaching a height of 20-40 cm, transgenic plants comprising the above described chimeric gene are sprayed with the inducer compound BTH to elicit expression of the *Amb*

a I.1 gene. Plant material is harvested just prior to induction and at 8 hours and 1, 2, 3, 7, and 14 or 28 days following induction and flash frozen in liquid nitrogen.

The above disclosed embodiments are illustrative. This disclosure of the invention will place one skilled in the art in possession of many variations of the invention. All such obvious and foreseeable variations are intended to be encompassed by the appended claims.

***What is claimed is*:**

1. A plant comprising in its plastid genome a DNA molecule encoding a protein that is therapeutically active when administered to a host in need thereof in a therapeutically effective amount, wherein said plant is capable of expressing said protein.

2. A plant according to claim 1, wherein said protein is administered orally to said host.

3. A plant according to claim 1, wherein said protein is an antigen.

4. A plant according to claim 3, wherein said antigen is capable of suppressing or reducing an immune response or an inflammatory condition of said host against said antigen.

5. A plant according to claim 4, wherein said antigen is an allergen.

6. A plant according to claim 5, wherein said allergen is an airborne allergen.

7. A plant according to claim 5, wherein said allergen is a pollen allergen.

8. A plant according to claim 7, wherein said pollen allergen is selected from the group consisting of the ragweed allergens *Amb a I, Amb a I.1, Amb t V,* and *Amb a II*, the alder allergen *Aln g I*, the hazel allergen *Cor a I*, the ryegrass allergen *Lol p V,* the Johnson grass allergen *Sor h* and the birch allergen *Bet v I.*

9. A plant according to claim 5, wherein said antigen is selected from the group consisting of the cat antigen *Fel d I*, the dog allergen *Can f II*, the mosquito allergens *rAed a 1* and *rAed a 2*, the mite allergens *Der f I, Der f II, Der p I and Der p II*, bee venom allergen peptide PLA-2 and murine urinary proteins.

10. A plant according to claim 3, wherein said antigen is an autoantigen.

11. A plant according to claim 10, wherein said autoantigen is selected from a group consisting of collagen, myelin basic protein, myelin proteolipid protein, interphotoreceptor

binding protein, acetylcholine receptor, an S-antigen, insulin, glutamic acid decarboxylase, an islet cell-specific antigen and thyroglobulin.

12. A plant according to claim 3, wherein said antigen is a transplantation antigen.

13. A plant according to claim 12, wherein said transplantation antigen is a MHC protein.

14. A plant according to claim 12, wherein said transplantation antigen is a MHC class II protein.

15. A plant according to claim 12, wherein said transplantation antigen is a MHC class II a or b chain.

16. A plant according to claim 3, wherein said antigen derived from a pathogen, wherein said antigen is capable of immunizing said host against said pathogen.

17. A plant according to claim 1, wherein said protein is a blood protein, a hormone, a growth factor, a cytokine, an enzyme, a receptor, a binding protein, an immune system protein, a translation or transcription factor, an oncoprotein or proto-oncoprotein, a milk protein, a muscle protein, a myeloprotein, a neuroactive peptide or a tumor growth suppressing protein.

18. A plant according to claim 1, wherein said protein is an anti-sepsis peptide.

19. A plant according to claim 18, wherein said anti-sepsis protein is a bactericidal permeability-increasing protein.

20. A plant according to claim 1, wherein said immune system protein is an antibody.

21. A plant according to claim 1, wherein said DNA molecule is operably linked to a promoter capable of expressing said DNA molecule in the plastids of said plant.

22. A plant according to claim 21, wherein said promoter is a *clpP* promoter.

23. A plant according to claim 21, wherein said promoter is a 16S r-RNA gene promoter.

24. A plant according to claim 21, wherein said promoter is a transactivator-mediated promoter.

25. A plant according to claim 24, wherein said transactivator-mediated promoter is a T7 gene 10 promoter.

26. A plant according to claim 24, further comprising a heterologous nuclear expression cassette comprising a DNA sequence encoding a transactivator.

27. A plant according to claim 25, wherein said transactivator is a T7 polymerase.

28. A plant comprising in its nuclear genome a DNA molecule encoding a protein that is therapeutically active when administered to a host in need thereof in a therapeutically effective amount, wherein said therapeutically active protein is selected from the group consisting of the mosquito allergens *rAed a 1* and *r Aed a 2*, bactericidal permeability-increasing protein (BPI) and the pollen allergens *Amb a I, Amb a I.1, Amb t V, Amb a II, Aln g I*, *Cor a I*, *Lol p V, Sor h* and *Bet v I.*

29. A plant comprising in its nuclear genome a DNA molecule encoding a protein that is therapeutically active when administered to a host in need thereof in a therapeutically effective amount, wherein said therapeutically active protein is targeted to a subcellular organelle of said plant.

30. A plant according to claim 29, wherein said subcellular organelle is a vacuole.

31. A plant according to any one of claims 1 or 28 to 30, wherein said plant is a dicotyledonous plant.

32. A plant according to claim 31, wherein said plant is tobacco, tomato, soybean or spinach.

33. A plant according to any one of claims 1 or 28 to 30, wherein said plant is a monocotyledonous plant.

34. A plant according to claim 33, wherein said plant is maize or rice.

35. A plant according to any one of claims 31 or 33, wherein said plant is an edible plant.

36. A plant according to any one of claims 1 or 28 to 30, wherein the expression of said protein in said plant is chemically regulatable.

37. A plant according to any one of claims 1 or 28 to 30, wherein the expression of said protein in said plant is constitutive.

38. A plant according to any one of claims 1 or 28 to 30, wherein the expression of said protein in said plant is tissue-specific.

39. A plant according to any one of claims 1 or 28 to 30, wherein said host is a vertebrate.

40. A plant according to claim 39, wherein said vertebrate is a mammal.

41. A plant according to claim 40, wherein said mammal is a human, bovine, ovine, porcine, canine or feline.

42. A composition comprising a plant according to any one of claims 1 or 28 to 30 or plant matter derived from said plant, wherein said composition comprises a therapeutically effective amount of said protein.

43. A composition according to claim 42, wherein said plant is processed prior to being administered to said host.

44. A method comprising administering to an host in need thereof a composition according to claim 42 in an amount effective to improve the condition of said host.

45. A method according to claim 42, wherein said composition is administered orally to said host.

46. A method according to claim 45, wherein said protein is an antigen, whereby an immune response of said host against said antigen is suppressed or reduced.

47. A method according to claim 46, wherein said antigen is an allergen, an autoantigen or a transplantation antigen.

48. A method of treating or preventing a disease, comprising administering to an host in need thereof a therapeutically effective amount of a plant according to any one of claims 1 or 28 to 30 or plant matter derived from said plant.

49. A method according to claim 48, wherein said disease is an allergy, an autoimmune disease or the rejection of a transplantation.

50. A method according to claim 49, wherein said therapeutically effective amount is administered orally to said host.

51. A plastid transformation vector comprising a DNA molecule encoding a protein that is therapeutically active when administered to a host, wherein said DNA molecule is operably linked to a promoter capable of directing the expression of said DNA molecule in a plastid of a plant.

52. A plastid comprising a transformation vector according to claim 51.

53. A plant cell comprising a plastid of claim 52, wherein said plant cell is capable of producing said protein.

54. A plant comprising a plant cell according to claim 53.

55. A method comprising transforming the plastid genome of a plant with a plastid transformation vector according to claim 51.

56. A method according to claim 55, further comprising expressing a therapeutically effective amount of said protein in said plant.

57. A food product comprising an edible portion of a plant comprising a DNA molecule of any one of claims 1 or 28 to 30, wherein said food product is therapeutically active when administered to a host in need thereof in a therapeutically effective amount.

58. An agricultural product derived from a plant or plant part comprising a DNA molecule of any one of claims 1 or 28 to 30, wherein said agricultural product is therapeutically active when administered to a host in need thereof in a therapeutically effective amount.

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<210> 33
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<220>
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<220>
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